MULTIDIMENSIONAL APPROACH TO INVESTIGATING NEURAL MECHANISMS OF ANXIETY OUTCOMES FOLLOWING TRAUMATIC BRAIN INJURY IN MICE

by

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Abstract

Affective dysfunction including anxiety disorders are a major consequence of traumatic brain injury (TBI). However, much remains to be understood about the underlying neural signaling mechanisms. A lack of consensus in animal studies regarding the affective sequelae of TBI has been a major hurdle that has slowed progress, with studies reporting increase, decrease, as well as no change in anxiety following injury. Here, we addressed this issue directly in two series of experiments in mice following moderate to severe controlled cortical impact (CCI) injury. In the first, we examined the impact of injury on anxiety outcomes with a battery of different behavioral assays of anxiety as well as multiple time points (over 2 months post-injury), adopting the traditional approach of comparing the injured group with sham controls. Results from our experiments showed that the effect of injury is both time- and task-dependent, highlighting the importance of a multidimensional approach to studying anxiety following injury. In the second, we examined the role of individual variability in the response to injury. Specifically, we hypothesized (a) that there is substantial variability in the responses of individuals to TBI leading to a range of anxiety levels post-TBI, and (b) that comparison between extreme responders to TBI (rather than between TBI and sham controls) would lead to key insights into neural mechanisms of anxiety following injury. To test these hypotheses, we developed a novel approach that reliably identified animals either vulnerable or resilient to injury using the multidimensional behavioral profiles measured for each animal over time. This approach employed a combination of principal components analysis (PCA), unsupervised clustering, and behavioral validation to reliably identify animals either vulnerable or resilient to TBI. Immunostaining experiments in key areas of the corticolimbic network revealed robust multi-molecular signatures (of GABA, VGLUT and NPY expression) of vulnerability to anxiety

following injury. Notably, the extent of vulnerability to anxiety was tightly correlated with the changes in molecular expression across vulnerable individuals. Taken together, this work proposes a novel, multidimensional approach to the study of anxiety outcomes following injury, that has the power to uncover reliable neural mechanisms underlying vulnerability to anxiety.

Keywords: traumatic brain injury, anxiety behaviors, controlled cortical impact, resilience, vulnerability.

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1. Introduction

Traumatic brain injury (TBI) is a significant health problem, which presents a high prevalence worldwide [1, 2] and causes severe physical, social, and economic impairments [3]. Patients who suffer from even a mild or moderate TBI, which corresponds to approximately 80% of all injuries, are at a higher risk than the non-TBI population of developing cognitive [4], motor [5], and psychiatric disorders [6]. In particular, anxiety-related disorders, such as generalized anxiety disorder and post-traumatic stress disorder, affect up to 70% of all TBI patients [7]. Therefore, it is imperative to understand the underlying neural mechanisms of injury linked to anxiety disorders to manage adverse outcomes and improve patients' quality of life.

Animal models of injury have been widely used, in particular, rodent models, to replicate human symptomatology, understand the neural mechanisms of injury, and develop better therapeutic interventions for TBI patients. Overall, studies aiming at anxiety outcomes of TBI in rodent models measure unconditioned anxiety in exploration-based tasks [8]. Experimental paradigms such as the elevated plus maze (EPM), elevated zero maze (EZM), light-dark box (LDB) and open field test (OFT), rely on the animals' natural, opposing tendencies to explore a novel environment, as well as that of seeking a safe, protected area. Anxiety-like behaviors, in these assays, are measured as the proportion of time an animal spends exploring an exposed, potentially dangerous, zone versus a more protected, safe, zone in the behavioral apparatus. Despite the high prevalence of anxiety disorders following TBI, animal models have not consistently reproduced its maladaptive affective outcomes, particularly regarding anxiety.

Studies measuring anxiety outcomes of TBI using these paradigms often find opposing, contradictory findings: some suggest that injury decreases anxiety-like behaviors, whereas others indicate an increase or no change in anxiety [9-14].

The wide range of anxiety outcomes in animal models of TBI suggest a complex scenario and imply that many factors influence our interpretation of the behavioral outcomes. A diversity of injury models and degrees of severity, behavioral assays, and time-points have been adopted in these studies, which may lead to different anxiety outcomes. In this chapter, we aim to compare studies that have measured anxiety-like behaviors following TBI in rodents and identify similar or contrasting effects across behavioral assays, time-points, injury models, and severity. This approach will help us to define consistent methods and identify alternative approaches to the study of anxiety outcomes following TBI, and serves as a basis to the following chapters. In chapter three, we will test how different behavioral assays and time-points affect the behavioral outcome of TBI and anxiety. In chapter four, we will show how vulnerability and resilience may play a crucial role in interpreting the anxiety outcomes of TBI. Combined, these studies will shed new light in the anxiety outcomes of TBI in animal models, highlighting the importance of adopting a battery of behavioral assays and taking individual variability into account.

2. Animal models of TBI

TBI is a complex neuropathology, defined by damage to the brain caused by external forces, such as rapid deceleration, explosions, and inertial or rotational impact [15, 16]. Primary and secondary injury processes combined are responsible for the degree of injury and subsequent outcome [17, 18]. Primary injury occurs at the moment of impact to the head and leads to tissue

damage, impaired regulation of cerebral blood flow, an increase in intracranial pressure, and metabolic dysfunction [17, 19]. The secondary injury is characterized by increased glutamate excitotoxicity, delayed axonal injury, inflammation due to cytokine and chemokine release, increase in reactive oxygen species and programmed cell death, such as apoptosis [18, 20].

One of the challenges in studying TBI is its complex symptomatology and variability in outcomes among patients. Thus, reliably reproducing the injury in an animal model is a crucial step. Because of its substantial heterogeneity, replicating all aspects of injury in a controlled and reproducible manner can be challenging. Several animal models of TBI have been developed over the past few decades, to try to replicate specific aspects of the injury, and they have been useful in understanding several neural markers of TBI. Here, we will briefly review the most commonly used TBI models in rodents and how differences in injury model and severity can confound anxiety outcomes following brain injury.

2.1 *Weight-drop injury (WDI)*

WDI produces a focal brain injury with a free-falling, guided weight hitting either the intact skull, in the closed-head Marmarou's, and Shohami's models [21, 22] or the exposed intact dura mater, in the open-head Feeney's model [23, 24]. The severity of the injury is controlled by adjusting the mass and height the weight falls. Open-head weight-drop pathologies include hemorrhage and necrotic cavity beneath the impact site [24]. Closed-head height drop injury causes loss in cell number of cortex and hippocampus [25], breakdown of the blood-brain barrier [16], and apoptotic cells both in the ipsi and contralateral sides of the brain [26, 27]. Weight drop

injuries, both in closed and open-head models, also cause motor, cognitive, and emotional deficits [28-31].

2.2 *Fluid percussion injury (FPI)*

FPI is induced by a piston rapidly hitting a reservoir of liquid, which generates a fluid pressure pulse that strikes the intact dura-mater, leading to a diffuse injury [32]. The severity of the damage is controlled by changing the height the piston is released into the fluid reservoir [33]. The craniotomy and subsequent injury can be made at the midline, for a central injury, or laterally above the parietal cortex [33, 34]. FPI causes a high degree of axonal injury, focal cortical lesion and hemorrhage under the injury site, and widespread cortical and subcortical damage [35], besides glial cell increase in the injury site [32]. FPI causes motor, cognitive, sensory, and affective deficits [36, 37].

2.3 *Blast-related TBI (bTBI)*

In bTBI, a shock wave is directed to the head, to simulate an explosion, and resemble the common injury type in modern warfare [16, 38]. bTBI is traditionally divided into four stages The primary injury refers to the effect of the shockwave, the secondary injury is caused by debris projected at high speed, the tertiary injury involves to the blast wind that causes displacement of the body, and the quaternary injury is produced by the heat and smoke that follows the explosion [.39]. The pathologies of blast injury include contusion, edema, hemorrhage, diffuse axonal injury, and neurodegenerative processes across the whole brain [40]. One challenge with this type of injury is the considerable variation in parameters, such as head position and distance to

the shock wave, that can directly affect the molecular, functional, and behavioral outcomes of injury. Blast injury causes motor, cognitive, and behavioral deficits that can be long-lasting [41-43].

2.4 *Controlled Cortical Impact (CCI)*

CCI is generated by an electromagnetic piston directed onto the dura mater [44]. The degree of injury is controlled by changing the velocity, depth, and dwell time, tuning injury severity from mild to severe. CCI reproduces several pathological markers of human TBI [45]. Even though it was initially developed as a focal injury [46], studies have consistently found it induces diffuse axonal damage to distal brain areas affected in human TBI, such as cortex, hippocampus, and thalamus [47]. CCI also induces the upregulation of microglia [48], apoptotic cells [49], and impaired cerebral blood flow [50]. Besides pathological changes, CCI produces cognitive, motor, and emotional symptoms, similar to those observed in human TBI [49, 51, 52].

All the injury models described here reproduce important, but often different aspects of clinical TBI. For instance, bTBI closely reproduces the biomechanics of injury often experience by military personal, while FPI produces a diffuse injury commonly experience during falls or car accidents. Despite being a focal injury, CCI produces both the biomechanical and pathological aspects of clinical TBI [16]. Besides, injury parameters are easily adjustable and injury is highly reproducible, with relatively low mortality rates [53]. Because of these factors, in the studies described in this thesis, we adopted CCI as the model to understand the anxiety sequelae of brain injury.

3. Severity of injury across TBI models and the impact on affective behavioral outcome

In clinical TBI, the severity of the injury is assessed by the degree of consciousness and structural damage to the brain [54]. A widely used method to determine consciousness is the Glasgow Coma Scale (GCS), or its improved version, the GCSE [55], a 15-points neurological scale that measures eye, verbal, and motor movements. A high score (13-15) indicates a mild injury, a score between 9 and 12 indicates a moderate injury, whereas a low score (8 or less) indicates a severe TBI [56].

In animal models, the severity of the injury is controlled by changing mechanical parameters on the injury device, which affect the degree of focal, diffuse, and mixed damage [16]. The degree of injury can be assessed by measuring neurological changes (i.e., righting reflex), physiological changes (i.e., weight loss), histological change (i.e., volumetric loss), or behavioral change (i.e., neurological severity score - NSS). Animal models of TBI divide the injury severity into mild, moderate, and severe [16]. However, there is no golden standard to determine injury severity that is applicable to all models [54]. One difficulty is the fact that, in humans, severity is determined by the level of consciousness immediately after the injury, but in animal models, due to anesthesia, this assessment is not always possible. Besides, in clinical TBI, the patients' verbal response is a metric of consciousness. Finally, many animal studies do not mention the severity of the injury, which complicates interpretations and make the translation to clinical TBI difficult. In this section, we will describe how the parameters change in each type of injury, depending on the animal model used. It is worth noting, however, that in general, there is no standard to determine injury severity, and different research groups tend to use different

parameters to generate mild, moderate, and severe injury, even when using the same animal and injury model.

For the CCI model, the severity of the injury depends on the depth and velocity the piston hits the dura-mater. For instance, Washington et al. (2012) produced CCI in C57 mice with different injury levels. Velocity and dwell time were constant at 5.25 m/sec and 100 ms, respectively. Severity was controlled by changing the depth the piston hit the dura-mater: 1.5 mm (mild), 2.0 mm (moderate), and 2.5 mm (severe). Tucker et al. (2017) also produced different levels of CCI in C57 mice. Velocity was constant at 5.0 m/s, which is similar to Washington and colleagues, but the depth for their mild and severe injury was 1.0 mm and 2.0 mm, respectively, which is smaller than what Washington and colleagues used to generate what they considered to be the same level of injury. For rats, Almeida-Suhett et al. (2014) generated a mild injury in Sprague–Dawley rats, using the parameters: velocity 3.5 m/sec, dwell time of 200 ms, and depth of 2.0 mm. Wagner et al. (2007) also produced CCI in Sprague-Dawley rats. They do not qualify the severity of their injury, but adopted a faster and deeper injury, compared to Almeida-Suhett and colleagues. Their injury was at a velocity of 4.0 m/s and depth of 2.7 or 2.9 mm, which could be classified, in comparison to Almeida-Suhett and colleagues, as a more severe injury.

Siebold and colleagues (2018) reviewed studies on CCI severity and presented some guidelines to determine CCI severity based on tissue loss, neurological severity score, and cognitive deficits. In this system, a mild injury should present no or minimal loss or deficit, and severe injury should present the most substantial loss and deficit [53]. They also recommend the surgical parameters to generate each type of injury: mild injury should have a depth <0.5 mm and velocity <4.0 m/s, mild injury should have depth between 1.0 mm and 1.5 mm and velocity

between 4.0 m/s and 5 m/s, and severe injury should have depth >2.0 mm and velocity >5 m/s [53]. In chapters three and four, we adopt a moderate injury level, according to these parameters.

In the weight-drop injury models, severity is manipulated by changing the gravitational force, the mass, and height the weight falls. For instance, Meyer et al. (2012) induced what they called a mild injury in Sprague–Dawley rats by dropping a 175 g weight from 42 cm height. Hsieh et al. (2017) generated different levels of injury by changing the height of a 450 g weight: 1.0 m, 1.5 m and 2.0 m for mild, moderate, and severe injury, respectively. Pandey et al. (2009) generated a closed-head injury in Wistar rats by dropping a cylindrical metallic weight of 400 g from 1 meter height. They did not qualify the severity of their injury; however, comparing it to the other studies, their injury can be classified as severe. In Swiss mice, Schwarzbold et al. (2010) generated mild, moderate, and severe injury by changing the weights used (10 g, 12.5 g, and 15 g, respectively), and keeping the height constant at 120 cm, but another study also in mice Tweedie et al. (2007) produced a mild injury in ICR mice using weights of 30 g or 50 g dropped from an 80 cm of height.

In the lateral fluid percussion injury model, severity is determined based on mortality rate in the first 24 h, and severity can be manipulated by changing the atmospheric pressure, according to a protocol by Kabadi et al. (2010,[57]). For example, Johnstone et al. (2015) produced a moderate injury in male Sprague-Dawley rats using a mean pulse of 3.06 atm; Jones et al. (2008) produced a severe injury in Wistar rats by adopting a pressure of 3.2 to 3.5 atm, and Shultz et al. (2011) produced a mild injury in Long-Evans rats by adopting a mean injury force of 1.2 atm [58]. Shultz and colleagues reported no mortality among injured animals; however, the other studies do not mention the mortality rate.

These differences in injury parameters make it challenging to compare behavioral outcomes among studies. In addition, differences in time-points, the strain of animals and behavioral assays adopted differ across studies. Thus, a contradictory and confusing picture emerges as we try to characterize the affective behavioral outcomes of TBI.

Almeida-Suhett et al. (2014), Ajao, et al. (2012) and Wager et al. (2007) all produced mild CCI in rats and found increased anxiety-like behaviors. However, Amoros-Aguilar (2015) produced a mild CCI in rats and found no effects on anxiety-like behaviors. In rats, however, Amorós-Aguilar et al. (2015) and Cutler, et al. (2006) found reduced anxiety-like behaviors following a mild CCI-like injury. Cutler and colleagues, however, treated animals with progesterone, and they conclude that the effect on anxiety was due to this treatment. Thau-Zuchman et al. (2018) produced a moderate CCI in mice and found increased anxiety-like behaviors, measured by decreased time in the open arm in the EZM, whereas Wakade et al. (2010) found increased ambulation in the OFT, measured by increase number of squares crosses, with no effect on anxiety-like behavior. Washigton et al. (2012) exposed mice to mild, moderate, and severe CCI and found decreased anxiety responses for all injury levels, but Tucker et al. (2017) exposed mice to mild or severe CCI and found increased anxiety responses on the OFT only for the severe injury group. However, they found decreased anxiety in the EZM, LDB, and marble-burying suggesting a task-dependent effect. To complicate matters further, Sierra-Mercado et al. (2015) produced a severe CCI in mice and found no effects on anxiety-like behaviors in the EPM, measured by time spent in the open arm, one week post-injury.

Other injury models show similar contradictory findings. Meyer et al. (2012) produced a mild weight-drop injury in rats and found increased anxiety-like behaviors in the EPM. However,

Pandey et al. (2009), who also produced a mild weight-drop injury in rats, found decreased anxiety-like behaviors in the OFT. Schwarzbold et al. (2010) characterized varying severity levels (mild, moderate, and severe) of weight-drop injury in mice. They found an increase in ambulation on the OFT, but increased anxiety in the EPM post-severe TBI, demonstrating that the effect of injury on anxiety-like behaviors significantly depend on injury severity.

The studies described above show that even for the similar level of damage and animal and injury models, anxiety outcomes are varied. However, this does not take into account the time-points tested. Studies adopt time-points as early as a few minutes post-injury [59] and as long as a year [60]. This broad range of time-points allows us to understand the short and long-term effects of TBI on anxiety, yet we lack the knowledge of what constitutes an early versus a late effect. A systematic study testing at which time-points the effect become long-term would shed light on this issue, and allow future studies to determine the adequate time-point to be tested.

4. Assays of anxiety used in TBI studies: time course of behavioral changes

Several behavioral assays have been used to measure anxiety-like behaviors following TBI. Specifically, tests that measure innate, unconditioned anxiety have been primarily adopted. Those assays, often called exploration-based tasks, rely on the animals' innate tendency to avoid open and bright spaces (the anxiogenic zones) and prefer closed, darker areas (anxiolytic zones). Anxiety, in those tests, is inferred by measuring how much the animal is willing to explore the different areas of the maze: more time spent in the exposed zone indicates low anxiety, whereas more time in the safe zone implies high anxiety [61, 62]. These behavioral assays provide straight-forward, quantifiable approaches to measure how exposure to the injury changes the

affective, anxiety-like behavior of animals following TBI. Furthermore, they serve as a substrate to measure molecular and functional changes that may underlie the behavioral changes. In the next section, we will compare the results across different behavioral tasks measuring anxiety-like behavior in animal models of injury, then discuss differences in injury models and injury severity.

4.1 Elevated Plus Maze

The elevated plus maze is one of the earliest behavioral assays to assess anxiety. It is formed by two intersecting, narrow runways, forming a plus shape, placed at a distance from the ground. Two of the opposing runways have high walls, called closed arms, whereas the other two runways have no walls, and are called open arms [63, 64]. Anxiety is estimated by the exploration of the open versus closed arms.

The effects of TBI in the EPM are quite variable. Some studies report an increase in anxiety in the EPM post-TBI, measured by a decreased exploration of the open arms, or reduced number of entrances to the open arm. Adult and immature rats, and mice exposed to a mild closed head weight drop injury presented increased anxiety in the EPM on day six [65, 66], eleven [67] day 22 [68] and day 30 [69] post-injury. Immature rats exposed to the same type of injury present increased anxiety in the EPM at day six [70]. Mice exposed to a repeated weight-drop injury presented increased anxiety on day 4 [71] and at one and two months post-injury [31] and mice exposed to CCI presented increased anxiety in the EPM 24h, 48h [72]. Rats exposed to LFP exhibited a decreased number of entrances and time in open arm in EPM between one and four months after injury [13, 73, 74] and increased anxiety after five repeated injuries 24h and

eight weeks post-injury [75]. Rats with an omega-3 deficiency presented increased anxiety seven days after an LFP [76], and rats exposed to an overpressure blast injury showed increased anxiety on day three [77, 78], nine [79], up to week 24 post-injury [80], measured by a decrease number of entrances and time in the open arm.

A few other studies, however, have reported a decrease in anxiety-like behaviors in the EPM, by observing a reduction in the exploration of the open arm, or increased time spent in the closed arm. Mice exposed to mild to severe CCI presented decreased anxiety 21 days post-injury [10] and mice exposed to stress paradigm plus CCI to model PTSD symptoms also presented a decrease in anxiety 25 days post-injury [81]. However, in this case, the effects of stress exposure may overlap with injury outcomes. Mice and rats exposed to a mild CCI or mild to severe weight-drop injury displayed an increased number of open arm entries and an increase in time spent in the open arm, 11 days, and nine weeks after the injury [82-84]. Siopi and colleagues exposed mice to a closed head weight drop and treated animals with minocycline, an antibiotic drug, a month after the injury. They found a small decrease in anxiety on the EPM three to seven weeks preceding treatment, measured by a slight increase in head dips [85]. In another study testing the effect of drug treatment, rats exposed to a cortical contusion injury and treated with progesterone up to six days later presented decreased anxiety, measured by increased time in the open arm [86].

Interestingly, several studies found no effect of injury on the EPM. Mice and rats exposed to CCI exhibited no change in anxiety-like behaviors in the EPM on one week, 28 days, and three to six weeks post-injury [87-89]. Juvenile rats exposed to a repeated weight drop injury were not different from controls three months post-injury [90]. Similarly, mice and rats exposed

to a single or repeated weight-drop injury presented no change in the EPM ten days to a year after injury [60, 91-95], and mice and rats exposed to single or repeated LFP presented no behavioral change in the EPM from one to twelve weeks after injury [75, 96-98].

Finally, Petraglia and colleagues found opposing effects on anxiety in the EPM, depending on the time-point measured. They exposed mice to a single or repeated closed-head weight-drop injury and found increased anxiety on day 14 for both single and repeated injury, but anxiety decreased one to six months later for animals exposed to repeated injury [99].

4.2 Elevated Zero Maze

The EZM is a circular version of the EPM, with two open and two closed quadrants, in which there is no ambiguous center zone as in the EPM [100, 101]. As in with the EPM, anxiety is estimated by the exploration of the open versus closed arms.

Fewer studies have adopted the EZM to measure anxiety-like behaviors following TBI, and they typically find increases in anxiety behaviors. Rats and mice exposed to a bTBI presented increased anxiety behavior, quantified as a decreased exploration of the open arm, measured as soon as 5 min after the injury [59], and lasting as long as 32 weeks [102]. Studies have found both increased and decreased anxiety behaviors following CCI. Male mice and juvenile rats exposed to a CCI presented increased anxiety behavior up to 60 days post-injury [9, 103, 104], and mice exposed to a weight-drop injury displayed increased anxiety behavior 21 days after the injury [67]. However, the opposite effect was observed using both male and female mice [105]: in two different studies, authors found decrease in anxiety behavior after CCI and weight-drop

injuries, respectively, up to 7 weeks post-TBI. Finally, some studies show no changes in anxiety behaviors at 90 days post-injury following a weight-drop injury in mice[85, 106].

4.3 Open Field Test

The OFT consists of a large box with high walls, and anxiety is inferred by the exploration of the exposed center versus the safe periphery of the arena [107]. In the OFT, studies in the effect of TBI on anxiety-like behaviors often report a decrease in time spent in the center of the arena, suggesting increased anxiety. This effect was observed in juvenile rats, adult rats, and mice exposed to CCI, which presented decreased in time spent in the center of the arena up to two months after the injury [9, 12, 105, 108-110]. Similarly, rats exposed to LFP and weight-drop injury presented increased anxiety up to 10 months after the injury [13, 111-113], and mice exposed to blast overpressure or weight-drop injury displayed increased anxiety up to two weeks later [59, 67, 114, 115]. A few different studies, however, have demonstrated decreased anxiety following TBI, measured by an increased exploration of the center of the arena. Rats exposed to LFP injury presented increased time in the center and increased number of crossings to the center of the OFT at one and three months post-injury [98], and mice exposed to CCI explored the center of the arena less ten days [116] and seven weeks after the injury [117].

A significant number of studies have found no effect of TBI on anxiety-like behaviors measured in the OFT. This effect has been observed in mice exposed to a mild CCI seven days [89], 30 days, and 12 weeks post-injury [108, 118]. Mice and rats exposed to a single [74, 75, 96] or repeated [97] LFP showed no change in anxiety up to 12 weeks after the injury. Similarly, mice exposed to a closed head concussion model showed no effect in anxiety behaviors up to

two weeks [119], and rats exposed to a blast overpressure TBI presented no change up to 17 weeks after TBI [102].

Studies also report conflicting results regarding locomotion changes in the OFT. Although this is not a metric for anxiety, it can be interpreted as an indirect measure of impulsivity [120] and used to determine whether locomotion deficits confound changes in anxiety. Immature rats exposed to weight drop presented decreased locomotion at day 22 post-injury [68], and mice exposed to the same model presented decreased ambulation at day five [71]. Rats exposed to a repeated closed-head weight-drop injury showed reduced activity measured by a decrease in total distance traveled up to three days for a mild injury and two weeks for a more severe injury [121]. They interpret this as an increase in anxiety not associated with motor or sensory deficit; however, animals exhibited a significant decrease in locomotion in the open field arena, which complicates the interpretation of the results. Rats exposed to an impact acceleration injury showed a decrease in distance traveled in the OFT arena at 24h, one week, and four weeks post-TBI [122], and decreased locomotion was also found in rats exposed to impact acceleration injury up to a month after TBI [123]. Finally, mice exposed to CCI and blast injury showed decreased locomotion in the OFT 48h post-injury [72].

Whereas some studies have reported decreased locomotion in the OFT, others have found hyperactivity in the arena, measured by increased distance traveled or increased number of crossings from different zones in the maze. Rats exposed to a closed head weight-drop injury presented increased locomotion at 25 to 27 days, with no direct effect on anxiety metrics [82]. The same was found in mice exposed to a CCI on day seven [106]. Other studies have found increased exploration of the arena one, seven and 14 days post-CCI in mice, with no effect on

anxiety [11, 118, 124]. Mice exposed to different levels of CCI (mild, moderate, and severe) exhibited increased locomotion up to 21 days post-TBI, accompanied by increased anxiety [105, 110, 125]. The same effect was found in rats exposed to a weight-drop model [112]. Perez-Garcia and colleagues found a small increase in locomotion in rats exposed to a blast injury up to 32 weeks post-TBI [102]. In another study, Yu et al. (2012) found that mice exposed to a CCI present increased locomotion accompanied by increased anxiety on day ten post-TBI [116]. Fromm and colleagues exposed rats to impact acceleration injury plus magnesium injection and found increased locomotion at six weeks post-injury [126]. They argue that these results suggest a decrease in anxiety in the treated group. However, given that several other studies report an increase in locomotion in the OFT post-TBI, with inconsistent results regarding anxiety, it is difficult infer that changes in locomotion, regardless of the direction, relates to change in the animals' anxiety state.

4.4 Light-dark box

The LDB consists of a small dark compartment and a spacious, brightly lit region. Anxiety is inferred by the amount of exploration of the aversive bright area versus the protected dark chamber [127]. Studies on the effect of TBI in the exploration of the LDB box consistently find increased anxiety after different injury models, and at diverse time-points. Rats exposed to a bTBI presented increase avoidance of the light chamber, both by decreased time spent and increased latency to first enter at seven days [128], 11 to 17 weeks [102], and 24 to 29 weeks after the injury [80, 129]. Similarly, rats exposed to a mild closed head injury present decreased time spent in the light compartment 48h and 30 days post-injury [130]. Mice exposed to a closed

head weight drop injury spent less time than shams in the light compartment 19 days after the injury [67]. One study, however, found the opposite result: male and female mice exposed to a severe CCI spent increased time in the light chamber two days after the injury; however, this was accompanied by decreased overall locomotion, which can confound the interpretation of the results [125].

4.5 Acoustic startle and prepulse inhibition

Besides exploration-based tasks, studies on anxiety and TBI have also used acoustic startle and prepulse inhibition. In the acoustic startle model, animals are exposed to bursts of noise and the intensity of the startle response, or muscle contractions the animal presents in response to the loud noise, is indicative of their arousal level [131, 132]. In the prepulse inhibition model (PPI), a weak prepulse sound anticipating the startling stimulus is capable of attenuating or suppressing the startle response. Importantly, the prepulse inhibition is not affected by habituation or extinction [133]. Animals who present higher anxiety will exhibit a decreased attenuation of the startle response, indicating heightened arousal.

Only a few studies have measured acoustic startle and prepulse inhibition following TBI to measure emotional arousal to an aversive stimulus. Those studies find opposing results. On the one hand, some have shown decreased startle response following TBI, which suggests decreased anxiety. Mice exposed to severe CCI and rats exposed to a bTBI showed a decrease in the startle response in the ASR model two to three weeks after the injury [10, 79]. However, other studies have found increased arousal to the acoustic stimulus and prepulse inhibition. Mice and rats

exposed to a bTBI also presented increased startle response up to eight months after injury [114, 129].

4.6 Fear conditioning

Fear conditioning, although not an anxiety metric, has also been often used to test emotional responses, as well as memory and learning, following injury [89, 90, 102, 134]. In the classical fear conditioning paradigm, an innocuous stimulus (CS), i.e., a tone, is paired with an aversive stimulus (US), i.e., a foot shock. After a few co-presentation of the two stimuli, the CS starts to elicit the innate response previously associated only with the US, i.e., freezing response. The proportion of time the animal freezes to both the CS and the context in which conditioning occurred are indicative of the animal's level of fear, and the animal's ability to remember the CS-US pairing [135].

Most studies adopting a fear conditioning paradigm and TBI try to emulate post-traumatic stress disorders, which is also a common neuropsychiatric disorder following injury [81, 102, 136]. However, there is no consensus on how close injury and fear exposure should be, and in which order they should be paired (TBI first, followed by fear conditioning, or vice-versa). Here, we summarize the studies that have adopted a fear conditioning paradigm in combination with TBI, regardless of the order or time-point used. For each study, we point out the temporal relation between injury and fear conditioning, and their findings.

Part of the studies demonstrates that animals exposed to different types of TBI and then trained on a fear conditioning paradigm show a decrease in fear expression during the testing phase. In one study, mice exposed to LFP were trained in a fear conditioning paradigm one week

or one month after the injury. At one week, there was no difference in freezing behavior between control and experimental groups during the training session, in response to both the context and the cue, indicating they were able to learn the CS-US association. Two days after conditioning, TBI animals presented less activity suppression when re-exposed to the context than controls, indicating a weaker fear response. However, groups did not differ in response to the cue when tested one week later. By one month, both groups presented the same levels of activity suppression to both the context and the cue, indicating a transient effect of the injury in contextual fear [137]. Rats exposed to LFP and trained in a fear conditioning paradigm five days later presented a marked decrease in freezing when they were re-exposed to the same context [138]. This effect was observed for as long as 42 days after the injury [139]. One study exposed mice to CCI and tested them in a contextual fear conditioning task 28 to 29 days after the injury. Although there was no difference between the groups during training, TBI animals displayed a significant decrease in freezing to the context when they were re-exposed 24h after training [140].

Another study exposed mice to a mild CCI and trained them in a fear conditioning paradigm three weeks after the injury, then re-exposed them to the same context and cue 24h and six weeks later. TBI animals presented typical acquisition, compared to both sham and naive controls. However, 24h later, in the testing phase, TBI animals exhibited significantly less freezing to the cue. After groups went through full extinction, they were re-exposed to the cue. TBI animals presented a resurgence of their fear response, measured by a significant increase in their freezing behavior, and indicating a long-term extinction deficit in those animals [84]. In another study, mice were exposed to repeated blast injury and trained on a fear conditioning

paradigm 23 weeks after the injury. Repeated TBI animals did not differ from sham in the proportion of freezing during acquisition, but they presented a significant reduction in freezing during cue and context re-exposure [39]. Genovese and colleagues trained rats in a food-maintained variable interval operant conditioning task, then in a fear conditioning paradigm. After conditioning, animals were exposed to three days of mild repeated bTBI. Animals were then tested to measure their conditioned response to the CS every week for two months. Animals exposed to bTBI presented a decreased expression of conditioned fear to the cue, measured by decrease in response suppression in the operant conditioning task [136]. In these examples, the acquisition was not impaired, which suggests no learning deficit. However, animals presented less freezing during re-exposure to the cue, which could indicate a sensory deficit, deficit in memory recall, decreased anxiety, or deficit in risk assessment.

Some other studies, on the other hand, have reported increased in fear acquisition and expression following TBI. Mice and rats exposed to a bTBI were trained in a fear conditioning task, eight weeks or 35 weeks after the injury. TBI animals did not differ from controls during the acquisition phase, but they exhibited increased freezing and slower extinction during the re-exposure phase for the cue test, and mice, but not rats, also froze more to the context compared to controls [102, 114]. Rats exposed to a fear conditioning procedure two to eight days after an LFP or a mild weight-drop injury presented increased and overgeneralized fear, measured by increased freezing to the context and the cue, measured by enhanced freezing on the test and extinction days, with no difference between groups in the training day and regardless of the intensity of the foot-shock [141, 142]. Mice exposed to mild CCI were tested in a fear conditioning paradigm 14 days after the injury. They presented enhanced fear during acquisition

and context extinction, as well as increased freezing after the reinstatement of the CS-US pairing following extinction [143]. Mice exposed to CCI and trained in a fear conditioning protocol at day 40 showed enhanced contextual fear measured by increased freezing on day 42, but no difference between groups in response to the cue [144].

Unpredictable stress or a social stressor have been adopted as a way to simulate the stressful condition some TBI patients experience. In one study, mice were exposed to unpredictable stressors for 21 days, and they were trained in a fear conditioning paradigm on day 12, during stress exposure. On the same day, they were exposed to mild CCI. Nine days after the injury, they were re-exposed to both the context and the cue used during fear conditioning. There was no difference between TBI only, TBI with unpredictable stress, and the control group during context testing, but animals exposed to stress combined with TBI presented increased freezing to the cue compared to controls [81]. Davies and colleagues exposed rats to a social defeat procedure, shortly followed by a mild weight-drop TBI to emulate the stressful condition that is a hallmark for PTSD. They were then tested in a fear conditioning paradigm seven days after the injury. Groups did not differ during acquisition, but animals that were exposed to the injury alone or social defeat combined with injury presented more freezing during the first day of re-exposure to the context. On days two and three of extinction exposure, only animals exposed to social defeat with TBI presented enhanced freezing, indicating that TBI-only animals presented normal extinction compared to controls. [66]

In some studies, there was no difference between TBI and control animals during fear conditioning. Mice exposed to severe CCI and trained in a fear conditioning paradigm either 24h before the injury or two weeks after did not differ from sham animals in the percentage of

freezing to the cue, both during acquisition and when they were tested during the extinction phase 16 days after CCI [89]. In a different study, male and female mice were exposed to either single or repeated mild closed-head frontal impact or CCI and tested for contextual and cue fear conditioning four weeks after injury. There was no difference between TBI and controls during acquisition, cue, and context extinction, regardless of the type of injury [119]. Finally, mice exposed to different levels of CCI (mild, moderate, and severe) and tested on a fear conditioning protocol 38 days later did not differ in the acquisition, context and cue extinction [145].

4.7 Differences in behavioral metrics

An issue that these results highlight is that even when the same behavioral assay is used, different metrics of anxiety behavior can be used in reporting the results. For instance, Jones et al. (2008) report an increase in the number of crossings to the center of the OFT arena after an LFP in rats suggesting decreased anxiety. Ajao et al. (2012) reported increased anxiety in the OFT measured by the decrease in the total distance traveled in the first 3 min in the OFT in rats exposed to CCI. Almeida-Suhett et al. (2014) reported increased anxiety measured by the decrease in the time spent in the center of the arena. While this is not a problem exclusive to TBI, but an overall challenge for translational models [146, 147], those differences add a layer of complexity when interpreting results across studies. Reporting change in distance traveled as a metric of anxiety is a particular issue, since it is not possible to infer the animal's anxiety level by its ambulation. Locomotion is a confounding factor for anxiety and can obscure the interpretation of the animal's internal state [148]. Therefore, it is important to report well-established and validated metrics of anxiety, such as the proportion of time in the anxiogenic

zone, as well as to report metrics such as the number of entrances, head-dips, and locomotion as additional or control measurements.

The ultimate goal of measuring behavioral differences is to understand the underlying neural mechanisms of injury that lead to these outcomes. In the next section, we will discuss some of the neural markers that have been utilized in studies of anxiety outcomes following injury.

Because of the complexity of TBI pathology, the effect of the injury changes over time. However, most studies measure anxiety after TBI only from the first few hours up to about a month after injury. For example, in the OFT, only a fifth of the studies shown in Table 1 tested anxiety for longer than a month after injury [9, 13, 73, 95, 102, 111, 126, 129]. While understanding the acute behavioral and neuropathological effects of injury is fundamental, there are crucial delayed structural and functional alterations that also need to be elucidated [52]. In clinical TBI, patients can suffer from psychiatric disorders for years after the injury [149]. Therefore, to bridge the translational gap from animal models to clinical application, and to develop appropriate treatments to help patients recover from the adverse effects of injury, more studies need to address the long-term effects of injury, as well as identify the behavioral and neurological progression of the injury.

5. **Neural markers**

TBI is followed by a series of morphological, structural, and physiological changes in the brain. Cell loss and volumetric changes in various brain regions have been associated with anxiety-dysfunction following TBI. Meyer and colleagues (2012) exposed rats to a mild weight-

drop injury, and they found that the increased expression of anxiety-like behaviors and conditioned fear was accompanied by a significant neuronal cell loss in the CA1 region of the dorsal hippocampus, besides increased cell number and volume in the amygdala nine days after injury [141]. Juvenile rats exposed to CCI showed a decrease in NeuN-positive cells near the injury and the ipsilateral and contralateral parietal cortex, indicating a cell loss in those regions. They also found a decrease in corpus callous size and decrease thickness of white matter tracks, measured by the neural process marker NF200. Those alterations in the neuronal number and axonal conduction underlie the behavioral changes following injury, more precisely, the increased anxiety two months after the injury [9]. Rats exposed to a moderate LFP displayed increased anxiety that was associated with a decrease in cortical volume in the barrel cortex, hippocampus, and corpus callosum, both in the ipsi and contralateral side, measure via MRI [13]. Ten-day old animals exposed to a weight-drop injury exhibited an increase in cell death and decreased brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), markers of neural growth and maturation, in regions CA1, CA3, and DG of the hippocampus, four days after the TBI, accompanied by decreased anxiety [70]. Another study observed that the increase in anxiety levels were accompanied by a decrease in neuron density in the amygdala, prefrontal cortex, and hippocampus, associated with high serum corticosterone levels, markers for stress response, three weeks after TBI [68].

TBI is a complex pathology that involves primary and secondary injury processes. While the primary injury encompasses the damage caused at the time of the impact to the head, the secondary injury involves several biochemical processes that lead to neural cell death, gliosis, white matter degeneration and axonal damage [150, 151]. One of the best-documented effects of

the secondary injury following TBI is an increase in oxidative stress in the hours following the injury [152]. Oxidative stress in the ipsilateral cortex following a moderate weight-drop injury model has been reported in mice 24h after the post-TBI, assessed by a significant increase in TBARS, a byproduct of reactive oxygen species activation. Two weeks after the injury, they found an increase in an anti-oxidative marker (GPx) in the ipsilateral hippocampus [83]. The molecular changes were accompanied by decreased anxiety, measure by an increase in exploration of the open arm in the EPM, 12 days after the injury. The increase in oxidative stress is a known secondary effect of TBI in the short term [153], and the increase in antioxidant markers can indicate a protective mechanism against the oxidative damage induced by TBI.

Another important sequela of TBI is neuroinflammation [154]. Rats exposed to a repeated mild LFP model showed an increase in microglia and macrophages, measured by increased ED1-labeled cells, a marker for inflammation in the injured cortex 24h after the injury, but not two months later [75]. Those changes were not accompanied by any effect on anxiety. Another study measured microglia activation and axonal damage after single or repeated injuries. By using silver degeneration staining, they found axonal degeneration in the external capsule and corpus callosum for both single and repeated injury. Iba-1 immunostaining, a microglia marker, showed an increased activation bilaterally in the amygdala [90], indicating that the amygdala, a hub for emotional responses deep in the brain, even when not directly affected by the injury, can suffer significant changes that may underlie the observed alterations in anxiety-like behaviors. Axonal degeneration was also observed three to eight weeks after blast injury, accompanied by increased fear and anxiety [114]. This type of damage is a marker of diffuse injury, commonly observed in

mild human TBI. An increase in Iba-1–positive cells around the lesion site has also been reported following CCI in animals who displayed increased anxiety [104].

Other inflammation markers have also been identified, such as increase in inflammatory cytokine, interleukin-1b (IL-1b) 12h and 24h after TBI, as well as increase in GFAP, a hallmark of gliosis, and OX-42, a marker for glial cell reactivity, in several brain regions, including the hippocampus, amygdala, and insula, which was accompanied by increased anxiety [124, 155]. Animals injected with a glial-cell activation inhibitor (MN166) had their behavioral deficits reversed, suggesting a role of inflammation and gliosis on anxiety-like behavior post-TBI [155].

Of particular interest to this work are the changes in GABAergic and glutamatergic neurotransmitter systems. It is well established that these systems play a fundamental role in the development of anxiety disorders [156, 157] and they are disturbed after TBI [12, 86, 134, 158], Evidence proposes a central role of gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the nervous system, in the control of anxiety, suggesting GABA dysregulation as a hallmark of anxiety disorders [159]. Inactivation of the basolateral nucleus of the amygdala by muscimol injection increases exploration of the anxiogenic zone in the elevated plus-maze, and the light-dark chamber [160], and decreased GABA-a receptor in the hippocampus, parahippocampus and orbitofrontal cortex leads to increased anxiety in the elevated plus maze, free-choice exploration and light-dark chamber, measured by an increased avoidance of the anxiogenic zones or novel object. [161]. Those studies point out that GABA deficiency in several limbic areas is linked to deficits in anxiety, whereas positive modulation of GABA, via GABA agonist injections, for instance, has an anxiolytic effect [162, 163]. A few studies have measured GABAergic transmission changes after TBI. Notably, Almeida-Suhett and

colleagues, who exposed rats to a mild CCI, correlated the increased anxiety observed in the OFT with hyperexcitability in the BLA, measured by loss of GABAergic interneurons and reductions in the frequency and amplitude of inhibitory postsynaptic currents (IPSCs). Besides the decrease in inhibitory signaling, they also found a significant increase in the surface expression of nicotinic acetylcholine receptor (a7- nAChR), implying an increase in the excitability of principal neurons within the BLA [12]. Another study also found decreased GABAergic signaling after a CCI-like injury, however, and importantly, in this study, animals were treated with progesterone and presented decreased anxiety in the EPM. Progesterone also led to a decrease in the edema caused by the injury, suggesting a protective role of progesterone after TBI [86].

The damage caused by the secondary injury often leads to excitotoxicity mediated by excessive glutamate release [164]. However, studies often find a downregulation of glutamate following TBI in animals who present alterations in anxiety behaviors, despite hyperexcitability of the amygdala being a hallmark of anxiety and fear [158]. Limbic and paralimbic structures, such as the amygdala, hippocampus, and medial prefrontal cortex are highly formed by glutamatergic pyramidal neurons [165]. Studies suggesting the involvement of glutamate in the control of anxiety show that glutamate antagonists injections into limbic brain areas, as well as intraperitoneal injections of mGLUR and NMDA antagonist, have an anxiolytic effect [128, 166, 167]. However, many studies in TBI have found a down-regulation in glutamatergic signaling, indicating an increased inhibition, which was often accompanied by increased anxiety. Mice exposed to a CCI presented changes in the excitatory marker PSD-95, which is involved in the maturation of excitatory synapses and promotes glutamatergic transmission. A decrease in

PSD-95 expression in the ipsilateral hippocampus was found beginning seven days post-injury. The loss of PSD-95 was also accompanied by a decrease in NeuN staining.; and correlates to the behavioral deficits, in particular to this review, the increase in locomotion in the OFT [118]. Animals exposed to CCI and blast injury also presented a decrease in PSD-95 [104] and a decrease in excitatory neurons in the BLA, measured by the expression of Thy1+ pyramidal neurons [114], all accompanied by increased anxiety. Mice exposed to a moderate CCI presented increased excitability in the lateral amygdala three months after the injury, assessed by the an increase in spontaneous excitatory postsynaptic currents (sEPSCs), suggesting increase in glutamatergic signaling [168]. Nevertheless, another study caused a weight-drop injury in 10-day old animals, and observed a decrease in N-methyl-D-aspartate receptor (NMDAR) immunoreactivity four days after injury, [70]. Contradicting those results, Reger et al. (2012) exposed rats to fear conditioning and concussive brain trauma and observed the upregulation of excitatory NMDA receptors in the BLA and the hippocampus. Behaviorally, these animals presented an increase in fear conditioning and over-generalization of fear to a novel stimulus [134].

Those results suggest that hyper- and hypo-excitability caused by the imbalance between GABA and glutamate in limbic areas may play a vital role in the control of anxiety in the murine brain. How the balance between these two neurotransmitters is affected by the injury concerning anxiety remains unknown.

6. Challenges in comparing studies: differences and common factors

Anxiety outcomes of TBI in animal models continue to present a series of challenges to researchers, due to the considerable complexities in modeling and measurement. Table 1 presents a summary of all the findings discussed above, demonstrating how heterogeneous the anxiety outcomes of TBI are. Factors such as differences in injury model and severity, behavioral assays and time-points, all play a role in the observed discrepancies in anxiety outcomes.

Table 1: summary of studies on TBI and unconditional anxiety outcomes

Behavioral Assay	Injury model/ animal	Effect on anxiety	Time-point where effect was found	Citation
	CCI rats	↑anxiety	7 days	Almeida-Suhett, 2014
			7 and 14 days	Wagner, 2007
			60 days	Ajao, 2012 (increased ambulation)
	CCI mice	↑anxiety	25 days	Yu 2012 Ojo, 2014
			3 weeks	Hsieh, 2014
			5 days	Desai (omega 3 deficient animals)
		↑ locomotion	24h, 7 and 14 days	Budinichi
			72h	Kimbler 2012
			Days 7, 14 and 21	Tucker 2016
			24h, 10 and 20 days	Tucker 2017
			7 days	Amenta, 2012
				Bajwa, 2016
				Wakade, 2010

Behavioral Assay	Injury model/ animal	Effect on anxiety	Time-point where effect was found	Citation
OFT		↓ locomotion	24h and 48h	Chauhan, 2010
		no effect	days 9 to 14	Wagner,2007b
			7 days	Sierra-Mercado, 2015
			30 days	Sherman Watanabe 2013
	WDI rats	↑anxiety	48h, 72h 1 and 2 weeks	Bolouri H, Sa¨ljo (repeated injury)
			Day 4	Singh et. al 2016
	WDI rats	↑ locomotion	25-27 days	Pandey, 2009
			3 months	Corrigan, 2017
		↓ locomotion	22 days	Baykara 2013
	WDI mice	↑anxiety	Day 5	Broussard 2017 (repeated injury)
			Day 9	Kosari-Nasab, 2018
	Closed head CCI mild	no effect	2 weeks	Cheng, 2014
	LFP rats	↑anxiety	12 weeks	Johnstone, 2015
			8 months	Rowe et. al 2016
			1 and 3 months	Jones, 2008
		no efect	24h and 48h	Schultz, 2012b
			1 day	Webster, 2015 repeated injury
				Schultz, 2015
	bTBI rats	↓ locomotion	28-32 weeks	Perez-garcia
		no effect	11 to 17 weeks	Perez-Garcia, 2018
	bTBI mice	↑anxiety	2 weeks	Heldt 2014
			?	Patel, 2014

Behavioral Assay	Injury model/ animal	Effect on anxiety	Time-point where effect was found	Citation
		↑anxiety	1h, 24h and 10 days	Xie, 2013
	Impact acceleration rats	↓ anxiety	6 weeks	Fromm, animals treated with magnesium
		↓ locomotion	4 weeks	Vink, 2003
			24h, days 7 to 28	O'connor
	LFP mice	no effect	7 days	Ferreira, 20014
	LFP rats	↑anxiety	Day 4	Kim 2017
		↓ anxiety	24h	Day
EZM	**CCI rats**	↑anxiety	Day 60	Ajao, 2012
	CCI mice	↑anxiety	Day 45	Thau-Zuchman 2018
			Day 14	Tchantchou, 2014
		no effect	3 months	Bajwa, 2016
		↓ anxiety	Day 21	Tucker 2017
	bTBI rats	↑anxiety	28-32 weeks	Perez-garcia
			11-17 weeks	Perez-Garcia, 2018
	bTBI mice	↑anxiety	?	Patel 2014
			5m	Xie, 2013
	WDI mice	no effect	7 weeks	Siopi
	WDI rats	↑anxiety	Day 21	Kosari-Nasab, 2018
	CCI mice	↑anxiety	24h and 48h	Chauhan, 2010
		↓ anxiety	Day 20	Washington, 2012
			Day 23	Ojo, 2014 for stressed animals
		no effect	1 week	Sierra-Mercado, 2015

Behavioral Assay	Injury model/ animal	Effect on anxiety	Time-point where effect was found	Citation
EPM			Day 28	Watanabe 2013
	Closed head CCI	no effect	4 Weeks	Cheng, 2014
		↓ anxiety (repeated) ↑anxiety (single)	2 weeks, 1 and 6 months	Petraglia, 2014
	CCI rats	↓ anxiety	Day 6	Cutler, 2006 (progesterone treatment)
		no effect	Week 3 and 6	Amoros-Aguilar, 2015
		↑anxiety	Day 6	Davies et, al 2016
	WDI rats	↓ anxiety	Day 25-27	Pandey, 2009
		↑anxiety	Day 6	Meyers, 2012
			Day 60	Sonmez, 2015
			Day 22	Baykara 2013
		no effect	Day 90	Fidan repetitive injury
			3 months	Corrigan, 2017
	WDI mice	↓ anxiety	Day 11-12	Schwarzbold, 2010
		↑anxiety	Day 4	Broussard 2017 (repeated injury)
			Day 30	Lital Rachmany, 2012
				Baratz, 2010
			Day 30 and 60	Yang 2015
			Day 11	Kosari-Nasab, 2018
		no effect	Day 7, 30 and 60	Zohar, 2011
			12 months	Mouzon 2014
			Day 9	Nichols, 2015

Behavioral Assay	Injury model/ animal	Effect on anxiety	Time-point where effect was found	Citation
			24h and 7 days	Moojen, 2012
		? check	3.5 weeks	Siopi
	LFP rats	↑anxiety	1 and 3 months	Jones, 2008
			24h and 8 weeks	Schultz, 2012
			12 weeks	Johnstone, 2015
			1 week	Tyagi (omega 3 diet)
			12 weeks	Schultz, 2015
		no effect	24h and 48h	Schultz, 2012b
			12 weeks	Webster, 2015 (repeated)
			Day 7	Day
	LFP mice	no effect	7 days	Ferreira, 20014
	bTBI rats	↑anxiety	Day 3	Adams, 2015
			24 weeks	Elder, 2012
			Day 9	Awwad
			Day 3	Sweis
ASR	**CCI mice**	↓ anxiety	Day 20	Washington, 2012
	bTBI mice	↑anxiety	Week 6-8	Heldt 2014
	bTBI rats	↓ anxiety	Week 2	Awwad
		↑anxiety	34 weeks	Perez-Garcia, 2018
LDB	**bTBI rats**	↑anxiety	24 weeks	Elder, 2012
			Day 7	Sajja
			11 to 17 weeks	Perez-Garcia, 2018
	CCI mice	↓ anxiety	Day 2	Tucker 2017
	WDI mice	↑anxiety	Day 19	Kosari-Nasab, 2018

Clinically, anxiety is recognized as a complex, multifaceted neuropathology that manifests itself in a range of forms, from generalized anxiety disorder to social anxiety and specific phobias [169]. Given this complexity, it is likely that in animal models, behavioral assays following TBI may be picking up on different facets of anxiety [170]. Besides concerns with sensitivity, and identification of state or trait anxiety, in these tests [62, 171, 172], behavioral measures in anxiety assays can also be confounded by changes in general activity, like locomotion, grooming, and eating [62]. Ramos (2008) defends the idea that emotionality, including anxiety, is multidimensional. In clinical anxiety, this is already recognized, and diagnostics guidelines, such as the Diagnostic and Statistical Manual (DSM), divide anxiety disorders based on distinct causes and environmental triggers. Extrapolating this logic to animal models of anxiety, Ramos (2008) proposes that anxiety varies in several independent axes, that are only accessible if we test animals in a battery of assays that bring about different contextual stressors (height, bright and open spaces, novelty, etc.).

Exposure to the injury itself, may be a complicating factor, by affecting the animals in ways that make traditional interpretations of their behavior in anxiety assays difficult. For instance, while human studies on sleep deprivation consistently point to an anxiogenic effect, animal models fail to replicate those findings, and often the results are contradictory [173]. Sleep researchers thus suggested that deprivation creates a complex phenotype, in which anxiety can co-occur with impulsivity or other disorders. In those cases, traditional anxiety metrics are not as informative. It is possible that TBI also leads to a complex phenotype, and that, in order to assess the effects of TBI on anxiety, we need an improved understanding of the anxiety assays applied to these models.

In addition, animals may present an individually variable response to the injury. This idea is well documented and accepted in clinical studies of TBI associated anxiety dysfunction. Patients who suffer TBI present different levels of vulnerability to anxiety following injury [7, 174]. Animal models of psychiatric disorders recognize this importance of individual differences among subjects [147, 175, 176]. In the field of stress, researchers have found that not all the animals exposed to a stressor will develop an anxious phenotype [175]. Therefore, they started to separate animals based on their behavior outcome after the exposure to a stressor. One of the most commonly used approaches to identify behavioral differences among stressed animals is the cutoff behavior criteria (CBC), developed by Cohen and colleagues [177-179], allowing for the identification maladapted and well-adapted subsets of the stressor exposed population [180]. Maladapted animals can be thought of as vulnerable to stress, while well-adapted animals are resilient to the effects of stress. This approach has allowed researchers to identify important behavioral, genetic and molecular differences between maladapted and well-adapted animals. For instance, different strains of rats present different levels of vulnerability to the stressor [178], vulnerable animals present higher levels of cortisol and HPA-axis activity [181], and early life stress increases the chances of animals to develop vulnerable phenotype later in life [182].

To summarize, we posit that there are three types of challenges to the interpretation of the anxiety outcomes of TBI in animal models. First, anxiety is likely a multifaceted pathology, and behavioral assays, when used individually, may fail to give a complete picture of the animal's emotional state. Second, the injury likely adds a level of complexity to the behavioral outcomes that are not captured without a range of behavioral assays. Third, the injury may have idiosyncratic effects on animals, leading to variability in terms of outcomes among animals.

To address the issues regarding the complexity of anxiety, some authors have proposed a multidimensional approach to anxiety, in which different behavioral assays are combined to capture different, but potentially overlapping aspects of anxiety [183, 184]. Ramos (2008) proposes that the animal models of anxiety most commonly used do present commonalities, but they are more effective when used in combination. Addressing the same issue, Cryan and Holmes (2005) argue that innate anxiety assays, although convenient for their ease-of-use, present challenges that may complicate interpretation of the results. They also propose the use of a battery of behavioral tests of anxiety, in order to increase reproducibility and better elucidate the animal's emotional state. We adopt this approach in the first part of our study, to characterize the time and task dependent response pattern of animals following a CCI model of injury. The diversity in injury models and severity of injury are integral to capturing the range of injuries that can occur in the real world. However, by keeping the differences among these in mind, using a diverse battery of assays, and measuring along both early and extended time-points, we can ascertain that a complete and accurate picture of the affective sequelae associated with TBI is captured.

Another important issue is that of variability in response to injury. When we take a closer look at the way animal studies are analyzed, we find that animal studies measuring anxiety response following TBI traditionally report the statistical comparison between TBI animals and their sham counterparts post-injury, and infer differences between the two groups (see examples: [12, 14]). However, such an approach is not informative about the individual responses to injury, and ignores the potential for the presence of sub-groups with different response patterns. To address this, we propose an approach inspired by the one adopted by the CBC: separating

animals exposed to TBI based on the effect the injury has on their anxiety levels, but generalize it to work on multidimensional behavioral data. We adopt a battery of behavioral tests and time-points, and use a dimensionality reduction method and clustering algorithm to identify vulnerable and resilient animals based on their behavioral profiles. Further, we study the relationship of several molecular markers in brain areas associated with anxiety behavior (mPFC, BLA, vHPC) with these behaviorally identified vulnerable and resilient subgroups.

Chapter 2: Methods and materials

1. Subjects

A total of 103 adult male C57BL6J mice (Jackson Labs, Bar Harbor, ME, USA) were used in the experiments. Animals belonged to two different batches. Table 2 summarizes the number of animals in each batch. Experiments reported in chapter three used the first batch of animals. Batch two was added for experiments presented in chapter four. Mice were split in two groups, CCI and sham, and the number of animals per batch are shown in Table 2. They were housed in colonies of four, with CCI and sham animals mixed in the same cage, in a 12 h light cycle (lights on from 7 am to 7 pm), with constant temperature and humidity (22°C and 40%). Food and water were available *ad libitum*. Animals' weight was monitored weekly, averaging 32 g. Mice were 6–8 weeks-old at the beginning of experiments and allowed 2 weeks of acclimation before experiments began. Behavioral testing was conducted between 10 am and 5 pm. This study was carried out in accordance with the recommendations of IACUC guidelines. All experimental procedures were approved by Johns Hopkins Animal Care and Use Committee.

Table 2: total number of animals per experimental batch, per condition (CCI and sham controls)

	Total number of animals	**Number of CCI animals**	**Number of sham animals**
Batch 1	42	25	17
Batch 2	61	46	15
Total	**103**	**71**	**32**

2. Injury Procedures

Animals underwent anesthesia under two different conditions. The first cohort of animals were anesthetized with Avertin (2,2,2 tribromoethanol - Sigma, St.Louis, MO) diluted in isotonic saline (500 mg/kg, i.p.), whereas the second cohort was anesthetized via 3% isofluorene. After a midline skin incision, a circular craniotomy was made midway between Bregma and lambda with the medial edge of the craniotomy 0.5 mm lateral to the midline. The mice were then subjected to a moderate to severe [14, 118, 185] CCI injury using a convex impactor tip of 3 mm in diameter. The injury was generated using the following parameters: 4.5 m/s velocity, 1.50 mm depth of penetration and a sustained depression for 150 ms. Mice were given 1 ml saline and 100 µl of 10% Meloxicam subcutaneously. Body temperature was maintained at 37°C with a warming blanket until full recovery from anesthesia (recovery of righting reflex). Sham-operated controls underwent the same surgical procedures with the exception of the traumatic injury. After surgery, mice were kept in individual cages for 72 h and monitored daily, then returned to their home-cages. Survival rate for the surgeries were about 90%. Righting times for both groups for recovery from anesthesia and surgery were similar (CCI: 101 ± 7.09 min; Sham: 103 ± 1 9.17 min). All surviving animals (71 CCI group, 32 sham control group) underwent further behavioral testing following recovery.

3. Behavioral Tests and Apparatus

Baseline and post-injury behavioral testing were performed with all mice - TBI and sham - together, in the same order each week. Testing proceeded as follows: animals were brought into the experimental room at least 30 min before the experiments began. They were first tested in the

OF, and given at least 2 h to recover before testing on the EZM. Twenty-four hours later, they were brought back to the experimental room and tested in the EPM. Mazes were cleaned with 70% ethanol between each animal. Each mouse was tested twice in each maze before injury. Baseline testing was performed 5–7 days apart, and injury was induced one week after the last baseline test. Baseline values were averaged for each animal to calculate their individual baseline level of anxiety.

3.1 Open-Field Test (OF—Accuscan, Columbus, OH)

The apparatus consisted of a 40.6 cm × 40.6 cm sound-attenuating box, with sensors on the bottom that monitored the animals' movement. A computer connected to the device recorded time spent in the center and distance traveled. Mice were placed in the center of the field and allowed to freely explore for 20 min. There were two trials before injury and four biweekly trials after injury.

3.2 Elevated Plus Maze (EPM) Test

Apparatus consisted of two intersecting runways (50 cm × 50 cm × 5 cm), placed at 1 m from the ground. One runway had no walls (two open arms), while the other had 20 cm dark, high walls (two closed arms). Mice were placed in the center of the maze, facing one closed arm, and allowed to explore for 10 min. There were two trials before surgery and four biweekly trials after. A camera placed above the maze recorded the animal's movement, and trials were analyzed using Ethovision© software. Behavioral metrics monitored were total distance traveled, distance traveled, number of entrances and time spent in each arm.

3.3. Elevated Zero-Maze (EZM) Test

Apparatus consisted of a circular platform (width: 5 cm, inner diameter: 40 cm), placed at 1 m from the ground and divided in four quadrants. Two opposite quadrants had a 20 cm dark, high walls (closed arm), while the other two had no walls (open arms). Mice were placed facing the entrance of a closed arm, and allowed to explore for 10 min. There were two trials before and four biweekly trials after surgery. A camera placed above the maze recorded the animal's movement, and trials were analyzed using Ethovision© software. Behavioral metrics monitored were total distance traveled, distance traveled, number of entrances and time spent in each arm.

4. Anatomical Metrics and Immunohistochemistry

4.1 Fixation and Sectioning

Two weeks after behavioral testing was finished, mice were deeply anesthetized (2.5% Avertin, 250 mg/kg body weight, i.p., Sigma, St. Louis, MO, USA) and transcardially perfused with 1 M phosphate buffer saline (PBS, 50 ml) followed by 4% paraformaldehyde (PFA, 100 ml, Sigma, St. Louis, MO, USA). Brains were removed and post-fixed in 4% PFA (50 ml) for 72 h, then transferred to a solution of 30% sucrose in 4% PFA, where they were kept refrigerated until sectioning. Coronal sections (40 μm) of the whole brain were taken using a slide microtome (Leica Microsystems, model CM-1860).

4.2 Immunohistochemistry

The primary antibodies used and their concentrations were: anti-GAD65/67 (1:1000, EMD Millipore), anti-vGLUT (1:1000, Thermofisher), and anti-Neuropeptide-Y (1:000,

ABCAM). Mounted sections were washed 3 times for 10 min each wash in 0.1M PBS and then incubated in blocking solution. For GAD65/67 and vGLUT, we used a blocking solution containing 10% normal goat serum and 0.5% Triton X-100 in PBS for 2 h at room temperature. For NPY, sections were blocked with a Mouse on Mouse immunodetection kit (Vector Labs) to reduce endogenous staining, for 1h at room temperature. Slides were then be incubated with primary antibody, in 10% normal goat serum for GAD65/67 and vGLUT, and 5% mouse IgG blocking reagent for NPY, in 0.5% Triton X-100, for 72 h at 4° C. After incubation, the sections were rinsed three times in PBS for 10min, and incubated with the secondary antibody, with the appropriate blocking serum, 0.5% Triton X-100, for 2 h in room temperature. The secondary antibodies used were Alexa Fluor 488 (1:1000, Abcam) for GAD6567 and vGLUT1, and Alexa Fluor 405 (1:1000, Abcam) for NPY. Sections were washed three times for 10 min with PBS, and incubated for 30 min with NeuroTrace 640/660 Deep-Red Fluorescent Nissl (Thermofisher) and mounted with Vectashield antifade mounting medium (Vector Labs). Separated, consecutive sections were used for each antibody.

5. Imaging for Volumetric Measure

Fluorescent images of the brain in the peri-injury region were taken with an Axiozoom v16 microscope (Carl Zeiss, Germany) using a 10× objective. Volume estimation was performed as described previously [186], adopting Cavalieri estimation [187]. Images from four sections were taken for each mouse in each area. We determined the position of each section by using Mouse Brain Atlas coordinates [188]. Sections were located between −0.70 and −2.46 mm Bregma, and they contained the lesioned area in the injured mice or equivalent position in sham

controls. BLA images were taken between −1.22 and 2.18 mm Bregma. Images were pre-processed using Fiji software (Schindelin et al., 2012). The hemispheric and BLA areas were outlined using Fiji on the ipsilateral and contralateral sides of the four brain sections, volume was calculated as the sum of the areas multiplied by the distance between sections (300 μm). The ipsilateral volume was normalized to the contralateral volume to estimate the extent of hemispheric volume loss (lesion) and change in BLA volume.

6. Imaging for Immunohistochemistry

Fluorescent images of the BLA, mPFC and vHPC were taken with a Zeiss LSM 700 microscope (Carl Zeiss, Germany) using 40x objectives. Four bilateral immunofluorescent labeled sections were taken in each mouse (8 images per animal per region, per marker), sections were approximately 200 um apart. Images were pre-processed using Fiji, and masks were generated for each image [189]. Number and intensity of puncta were calculated using a custom MATLAB analysis package (IMFLAN3D) [190]. A 'punctum' was defined as a cluster of 'connected' pixels identified in an unbiased manner using the MATLAB function bwlabeln with the 'eight-connected neighborhood' criterion.

7. Statistical Analysis

7.1 Experiment 1

For the behavioral tasks, a two-way unbalanced repeated measure ANOVA was performed after standard methods of outlier exclusion in MATLAB were applied. Data shown (at each time point in Figures 3.1, 3.2 and in Figures 3.3 and 3.4) represent distributions obtained

after removal of outliers following a standard procedure in MATLAB: samples that deviated from the median by more than fac*interquartile range were deemed outliers (average value of fac used = 1.5). *Post hoc t*-tests were performed whenever ANOVA indicated a significant effect, and *p*-values were corrected by a false discovery rate (FDR) test. For hemispheric volume, the normalized ipsilateral volumes (ipsilateral/contralateral) were compared among groups using *t*-tests and FDR correction. For GAD65/67 puncta analysis, the mean of each group was compared using *t*-test with FDR correction. Individual baseline data was averaged across the two pre-injury sessions to determine the mean value for each animal and assay. Post-injury behavioral data was plotted normalized to each animal's individual baseline: behavioral metrics at each time-point was divided by the animal's baseline value. Values greater than one indicate the mouse's anxiety level decreased in comparison to its baseline, whereas values smaller than one indicate increase in anxiety. This approach allows us to measure how anxiety changed for each individual animal because of the injury. Normalized values are presented as mean ± standard error (SEM). All statistical tests were performed using MATLAB (Mathworks Inc., Natick, MA, USA). Statistical analysis was considered significant for $p < 0.05$.

7.2 Experiment 2

For the behavioral tasks, a three-way unbalanced repeated measure ANOVA was performed. Post-hoc t-tests were performed whenever ANOVA indicated a significant effect, and p-values were corrected by a false discovery rate (FDR) test. For hemispheric volume, the normalized ipsilateral volumes (ipsilateral / contralateral) were compared among groups using three-way ANOVA, and t-tests with FDR correction were used whenever a significant difference

was found. For the puncta analysis, the mean of each group was compared using three-way ANOVA and t-test with FDR correction. Individual baseline data was averaged across the two pre-injury sessions to determine the mean value for each animal and assay. Post-injury behavioral data was plotted normalized to each animal's individual baseline: behavioral metrics at each time-point was divided by the animal's baseline value. Values greater than one indicate the mouse's anxiety level decreased in comparison to its baseline, whereas values smaller than one indicate increase in anxiety. This approach allows us to measure how anxiety changed for each individual animal because of the injury. Normalized values are presented as mean +- standard error (SEM). All statistical tests were performed using MATLAB (Mathworks Inc., Natick, MA). Statistical analysis was considered significant for $p<0.05$.

7.3 Principal component analysis and k-means clustering

PCA is a statistical procedure that uses an orthogonal linear transformation to convert correlated observations into uncorrelated variables (or principal components, PCs). Each PC explains a part of the variability in the dataset, with the first accounting for the most variability, and successive PCs accounting for progressively smaller amounts. We combined the behavioral metrics in the EZM and OFT on weeks one, three, five and seven, and EPM on weeks three, five and seven into one 11-dimensional vector, which we refer to as the animals' behavioral profile. We applied PCA to the dataset constituting the behavioral profiles of all the TBI animals, and found that the first four principal components together accounted for a majority (Cohort A: 92%, Cohort B: 87%) of the total variability in the dataset. We then applied a clustering algorithm (K-means) to identify two distinct clusters of animals (because we were interested in the extreme

responders, we asked the algorithm to cluster the data optimally into two clusters). For the 'control PCA', we performed an independent analysis (PCA and k-means) on the behavioral profile of sham animals.

7.4 Effect size (Eta-squared)

In order to determine the magnitude of the immunostaining results, following a three-way ANOVA and post-hoc testing, we calculated the effect size (eta-squared, η2) for each comparison between resilient, sham and vulnerable animals. Eta-squared was calculated using the Effect Size toolbox v1.6.1 [191], and the function *mes2way*, in Matlab ©, following the formula:

$$\eta^2 = \frac{SS_{effect}}{SS_{total}}$$

We report the strength of effect sizes follows: small (η2 = 0.02), medium (η2 = 0.06), and large (η2 = 0.14) [192].

7.5 Correlation analysis

We calculated the correlation between the behavioral data on the EZM on week seven with all the instances where we found a statistically significant difference between vulnerable animals and resilient or sham controls. We chose the EZM because it was the behavioral metric that presented the most consistent results, and week seven was the closest time-point to the immunostaining procedure. We calculated Person's linear correlation coefficient using the Matlab © function *corr*. We adopt the criteria of r>06 for a strong correlation, and 0.2<r>0.6 for a weak to moderate correlation [192].

Chapter 3: Long-Term Effects of Traumatic Brain Injury on Anxiety-Like Behaviors in Mice: Behavioral and Neural Correlates

1. Introduction

Traumatic brain injury (TBI), characterized as any damage to the brain caused by external acceleration or deceleration forces [15, 193], is a complex health problem affecting millions of people worldwide [2]. TBI produces considerable and wide-ranging losses in cognitive, motor and affective functions [194, 195]. This is true even of injuries considered mild or moderate, which constitute 80% of all cases and can lead to debilitating long-term effects [196, 197]. The high prevalence and substantial impact of TBI emphasize the importance of understanding the neural mechanisms underlying the outcomes of injury.

Animal models of TBI, and specifically rodent models, have been used to replicate the human symptomatology, examine neural mechanisms and test therapeutic interventions [16]. The cognitive and motor outcomes of TBI have been well established and replicated among pre-clinical studies [198-200]. However, affective outcomes, and specifically, maladaptive anxiety outcomes, which can affect up to 70% of all TBI patients [6, 7, 201, 202], have been difficult to reproduce in animal models. Studies in rodents, which typically quantify anxiety as the proportion of time animals spend in a more exposed, anxiogenic portion of a behavioral apparatus, as opposed to an enclosed, less anxiogenic zone, have yielded inconsistent and, at times, contradictory results [9-12, 88, 89, 108, 118, 203, 204].

One group of pre-clinical studies found an increase in anxiety-like behaviors following TBI. Injured rats and mice spent less time than sham controls in the anxiogenic zones in the open

field test (OFT), elevated plus maze (EPM), elevated zero maze (EZM) and in the dark-light chamber tests, and exhibited increased immobility in the tail suspension test for up to 8 weeks post-TBI [9, 12, 108, 203, 204]. Paradoxically, another group of studies found a decrease in anxiety-like behaviors, with injured animals spending more time than sham controls in the anxiogenic zones in the EPM and OFT for up to 3 weeks post-TBI [10, 11, 118]. Finally, a third group of studies found no difference between injured and sham animals in the EPM and OFT for up to 6 weeks post-TBI [88, 89, 203]. It is likely that the diversity in range of time-points tested - ranging from 3 to 8 weeks; and differences in the choice of behavioral tests administered to assess anxiety account for these variations. Together, they contribute to the idiosyncratic results reported in the literature. An additional factor that can add to variation is the degree of injury severity, which differs between studies. However, even for a similar injury level, variable results have been described. Wagner et al. (2007), Ajao et al. (2012) and Almeida-Suhett et al. (2014) produced mild controlled cortical impact (CCI) in rats and found increased anxiety at 7 days [12], at 14 days [108] and up to 60 days [9].

However, Amorós-Aguilar et al. (2015) produced a mild CCI in rats and found no effects on behavior. A moderate to severe CCI can lead to no anxiety early after injury [89], increased anxiety at 45 days [104], or variable effects depending on the test used [125]. Washington et al. (2012) explicitly tested this by using varying levels of injury severity. While severity impacted lesion volume differentially, behavioral effects at 3 weeks after injury were similar. Mild, moderate or severe injury leads to reduced anxiety in the EPM at 21 days post injury, and showed no effects in the OFT. However, moderate or severe injury showed greater lesion volumes and decreased ipsilateral hippocampal volumes, as compared to mild injury.

The primary goal of this study was to explicitly address the issues of variability in time-points and behavioral assays in mice. We hypothesize that there is a time and test dependance of anxiety-like behaviors measured following CCI. To achieve this goal, we used a well-established model of brain injury—the CCI model [16, 35], employed a battery of commonly used tests to measure anxiety-like behaviors in mice, and measured anxiety over a long time-course. Anxiety behaviors were assessed every 2 weeks up to 7 weeks following moderate to severe injury, and all mice were subjected to a battery of OFT, EPM, and EZM tests of anxiety at each time point. This experimental design permitted the direct comparison of different anxiety metrics within the same animals and over time, allowing for a systematic dissection of the behavioral affective sequelae of anxiety following injury. Repeated measures of anxiety tests, on one hand have been interpreted as leading to habituation in response. However, this is also associated with changes in assessment of threat in the anxiogenic zone and implies the development of a learned form of fear response [205], which has implications for anxiety behavior. Additionally, following this behavioral characterization, we also measured a molecular marker of GABAergic function in the basolateral amygdala (BLA), one of the key hubs of anxiety processing, to gain a window into the molecular underpinnings of changes in anxiety-like behavior following injury.

Our results support the central hypothesis of this study, by demonstrating that early effects on affective behavior following injury can differ from late effects, and that the observed effects can vary depending on the behavior test used. Consequently, they reveal a complex picture regarding the affective consequences of injury, and argue for the need for a standardized and comprehensive approach to study affective outcomes of TBI in animal models.

2. Results

2.1 Effects of TBI on Anxiety-Like Behaviors Exhibit a Complex Trajectory

We determined the long-term effects of TBI on affective outcomes in a battery of innate anxiety behavioral tests following exposure to a CCI injury. Mice were tested in the OFT, EZM and EPM tests. These tasks exploit the animals' natural conflict between seeking protection and exploring a novel environment. All mice were tested twice in the behavior tests before surgery, and these were averaged to determine each animal's baseline level of anxiety. Post-injury or sham surgery, they were then tested on weeks 1, 3, 5 and 7. Anxiety was measured by the change in proportion of time they spent in the anxiogenic zone in each assay, as well as number of entrances to the open arm (EZM and EPM). Values at each time point are normalized to each animal's baseline; this approach allowed us to determine how the anxiety level of each animal changed due to the injury. We controlled for possible locomotion deficits, by measuring total distance traveled in all three mazes.

Examples of heat maps representing the proportion of time TBI and sham mice spent in each zone in the OFT arena on week 7 are illustrated in Figure 3.1A. In this test, we found an overall effect of injury across the population, tested by an repeated two-way ANOVA ($F(1,3) = 4.04$, $p < 0.01$, Figure 3.1B). Injured mice spent significantly more time in the center of the arena on week 7 (*post hoc t*-test with FDR correction, $p = 0.02$), but we did not observe difference between groups in the other time-points. These results indicate that the main effect of injury was driven by the strong decrease in anxiety TBI mice presented on week 7.

In order to compare the effect of injury across different behavioral assays, we measured anxiety-like behaviors in the EZM and EPM tests. Heat maps illustrating the proportion of time mice spent in each type of arm are shown in Figures 3.1C, E. In the EZM, there was no main effect of injury in the proportion of time spent in the open arm (repeated two-way ANOVA, $F = 0.002$, $p = 0.96$, Figure 3.1D). However, we found a strong interaction effect between time and injury (repeated two-way ANOVA, $F(1,3) = 4.24$, $p < 0.01$). TBI mice display increased anxiety on week 1 (*post hoc t*-test with FDR, $p < 0.01$) and decreased anxiety on week 5 (*post hoc t*-test with FDR correction, $p = 0.01$), as measured by time spent in the open arms. Interestingly, in both the OFT and EZM, we observed a decrease in anxiety-like behaviors as compared to sham controls, on week 7 and 5, respectively, which suggests a delayed effect of the injury. There were no differences between groups in baseline, prior to TBI.

In addition to the OFT and EZM tests, we measured anxiety in the EPM. Figure 3.1F shows the proportion of time mice spent in the open arm in this assay. We found a main effect of injury, tested by a repeated two-way ANOVA ($F(1,3) = 4.44$, $p = 0.03$), and a strong effect of time (repeated two-way ANOVA, $F(1,3) = 9.18$, $p < 0.01$). *Post hoc t*-test with FDR correction showed that TBI mice spent significantly less time in the open arm on week 3, indicating increased anxiety ($p < 0.05$). We observed that both in the EZM and EPM tests, there was an increase of anxiety-like behaviors on earlier time-points: on week 1 for the EZM and week 3 in the EPM. Across the 3 behavioral assays, our results indicate that the effects of TBI on anxiety-like behaviors are time- and task-dependent: generally, TBI led to an early increase and late decrease in anxiety-like behaviors.

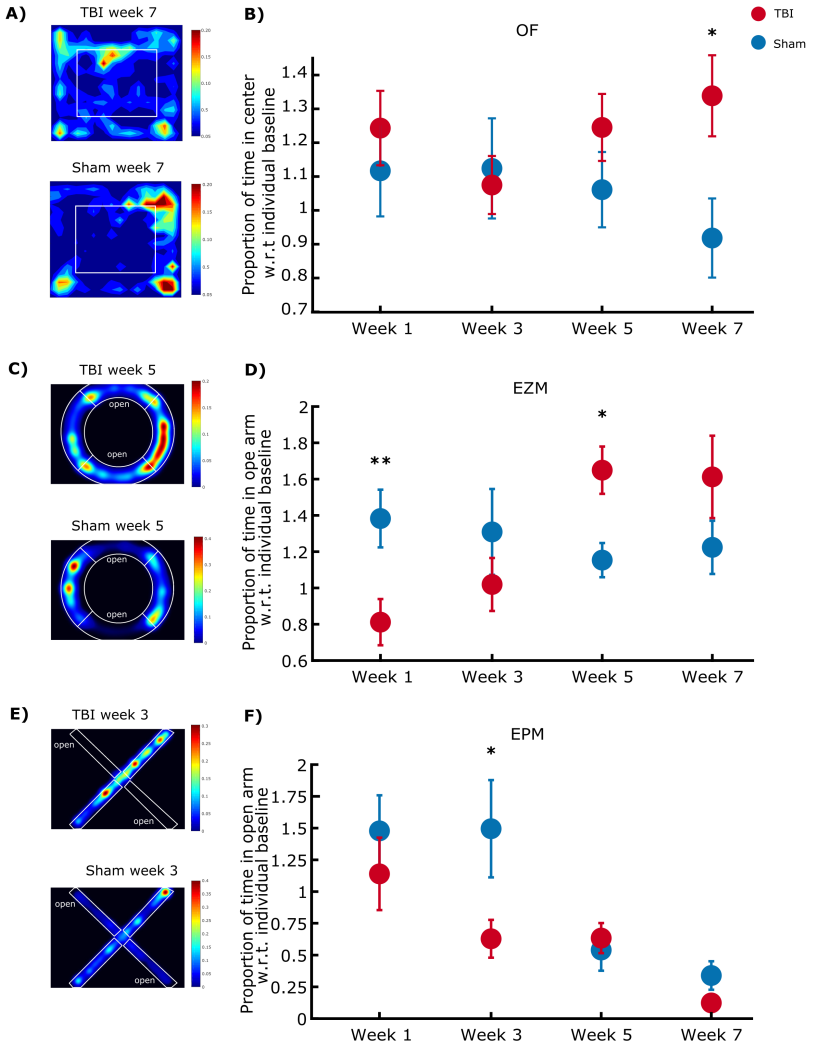


Figure 3.1. Traumatic brain injury (TBI) causes long-term effects on affective behaviors. **(A,C,E)** Representative heat maps of TBI and Sham animals in the open field (OFT), elevated zero maze (EZM) and elevated plus maze (EPM), respectively. Warmer colors represent that the animals spent more time on that zone. **(B)** Proportion of time in the center of the OFT arena. Each circle represents one mouse. Horizontal bars denote means; shaded regions denote SEM. There is a main effect of injury (repeated two-way ANOVA, $p < 0.01$) and anxiety significantly decreases on week 7. **(D)** Proportion of time in the open arm of the EZM; conventions as in **(B)**. There is an interaction between time and injury (repeated two-way ANOVA, $p < 0.01$). Anxiety is significantly increased on week 1 and decreased on week 5. **(F)** Proportion of time in the open arm of the EPM; conventions as in **(B)**. There is a main effect of injury and time (repeated two-way ANOVA, $p < 0.01$). Anxiety significantly increases on week 3. Panels **(B,D,E)** show data are from $n = 25$ mice in TBI condition, and $n = 17$ mice in sham condition after removal of outliers at each time point ("Materials and Methods" section); the number of outliers at any time point for any condition did not exceed four mice; $*p < 0.05$, $**p < 0.01$ by *post hoc* *t*-test with false discovery rate (FDR) correction.

As an additional metric of anxiety, we measured number of entrances to the open arm in the EZM and EPM. Injured mice did not differ from sham on number of entrances in the EPM (repeated two-way ANOVA, $F(1,3) = 0.25$, $p = 0.61$, Figure 3.2D). In the EZM, we observed an effect of injury in the number of entrances to the open arm (repeated two-way ANOVA, $F(1,3) = 5.10$, $p = 0.02$, Figure 3.2E), and TBI mice presented significantly fewer entrances on week 1 (*post hoc t*-test with FDR correction, $p = 0.01$), which is consistent with the decreased anxiety observed in the proportion of time in the open arm on week 1 in this maze.

To ensure that anxiety metrics were not affected by locomotion deficits, we measured total distance traveled in each maze. In the OFT, TBI led to hyperactivity throughout all time points (repeated two-way ANOVA, $F(1,3) = 36.69$, $p < 0.01$, *post hoc t*-test with FDR correction, $p > 0.05$, Figure 3.2A). Injury had no locomotion effect on the EZM (repeated two-way ANOVA, $F(1,3) = 0.56$, $p = 0.45$, Figure 3.2B) and EPM tests (repeated two-way ANOVA, $F(1,3) = 1.23$, $p = 0.26$, Figure 3.2C). Since TBI and sham did not differ in terms of total distance traveled in the EZM, we concluded that the reduced number of entrances to the open arm in this maze reflects the increase in anxiety those mice presented on week 1. Finally, we concluded that the effects in anxiety-like behaviors were not confounded by locomotion deficits.

Our behavioral results indicated that the effects of TBI in anxiety-like behaviors are complex. Although injury did not affect locomotion in the EPM and EZM tests, the effect on anxiety metrics varied across time-points and assays. By comparing different assays, we identified that in the first few weeks after injury, mice had a significant increase in anxiety-like behaviors. This effect, however, inverted after about a month post-injury. After this point, TBI mice display decreased anxiety-like behaviors as compared to sham controls.

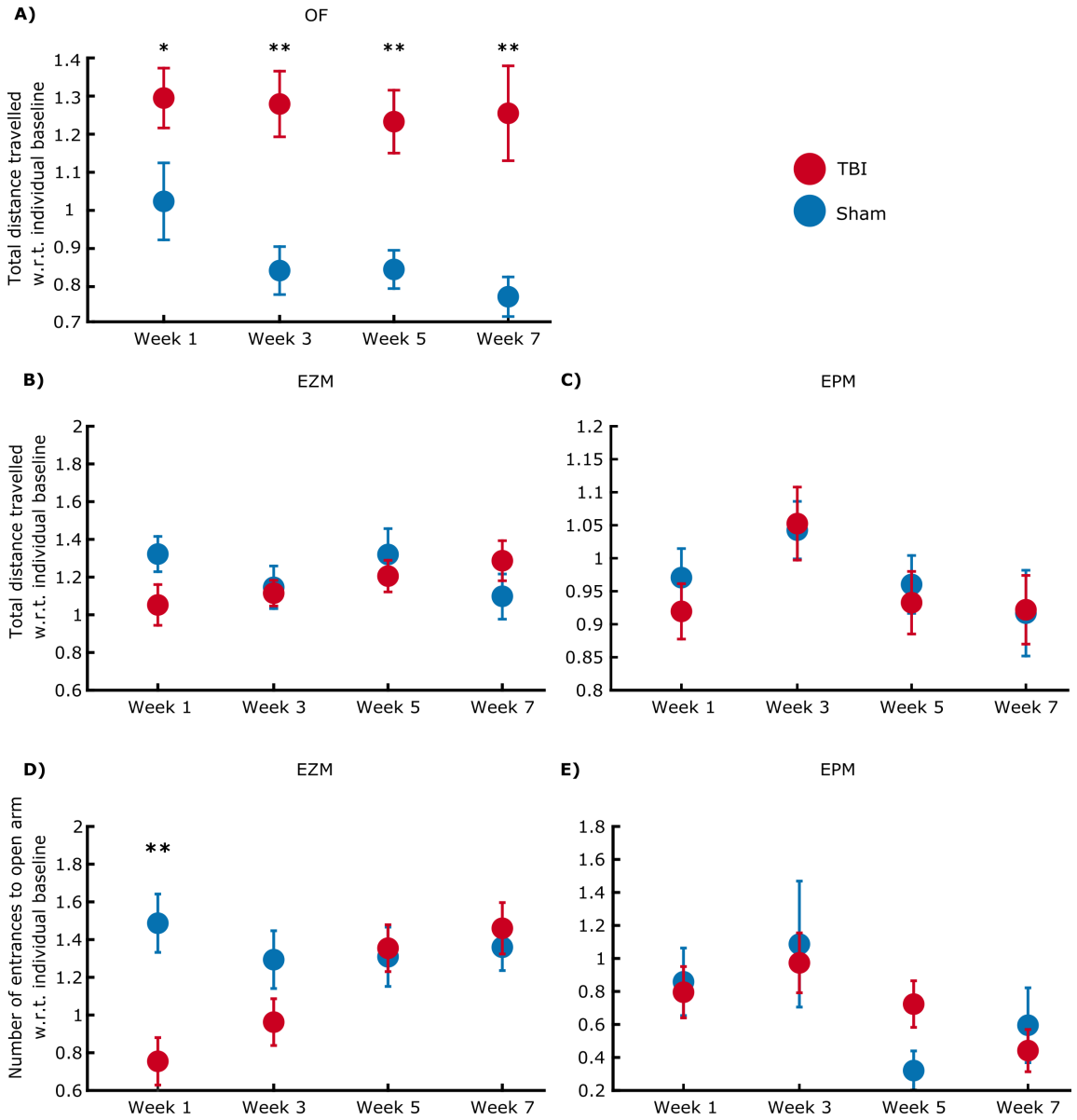


Figure 3.2. TBI effects vary across behavioral assays and metrics. **(A)** Total distance traveled in the OFT. There is a main effect of injury (repeated two-way ANOVA, $p < 0.01$), and TBI animals travel more at all time-points compared to sham controls. **(B,C)** Total distance traveled in the EZM and EPM, respectively. There is no effect of injury in locomotion in these assays. **(D)** Number of entrance to the open arm in the EZM. There is a main effect of injury (repeated two-way ANOVA, $p < 0.05$) and TBI animals present fewer entrance on week 1 than sham controls. **(E)** Number of entrances to the open arm in the EPM. There is no effect of injury. In all panels, each circle represents one mouse. Horizontal bars denote means; shaded regions denote SEM. Data from n = 25 mice in TBI condition, and n = 17 mice in sham condition are shown after removal of outliers at each time point ("Materials and Methods" section); the number of outliers at any time point for any condition did not exceed three mice; *$p < 0.05$, **$p < 0.01$.

2.2 Injury Was Consistent Across Mice

Considering the complex effects of injury, we asked if differences in the extent of injury across mice could be a confounding factor. Because we were interested in the long-term effects of injury, we sacrificed the mice on week 9 post-injury or sham surgery, obtained brain sections and quantified hemispherical volumes as an anatomical metric of the extent of injury.

Coronal brain sections of an injured mouse illustrating the extent of the injury to the cortex and hippocampus, as well as a brain section in the same region of control mouse are presented in Figures 3A,B. We determined the extent of the injury by measuring the hemispheric area of the peri-injury site, on the ipsilateral and contralateral sides of four brain sections per mouse, located between −0.70 and −2.46 mm Bregma, and multiplied the sum of the areas for each side by the distance between sections (300 μm; $n = 10$ for each group). The ipsilateral hemispheric volume of each brain was then normalized to its contralateral side. There is no effect of injury on the contralateral side (data not shown). The injury caused a reduction of the ipsilateral hemispheric volume of approximately 20%, compared to control mice (Figure 3.3C, two-tailed *t*-test, $p < 0.01$). The extent of the injury was consistent among TBI mice, as shown by the standard error of the mean (SEM TBI = ±0.04, SEM sham = ±0.01).

2.3 Injury Did Not Cause a Volumetric Change in the BLA

We proceeded to identify potential neural correlates of the long-term behavioral outcomes. Past work has demonstrated that injury can cause volumetric changes in brain areas distal from the site of injury. Motivated by this, we hypothesized that the affective behavioral changes could correlate with anatomical changes in the BLA, a key brain area involved in the

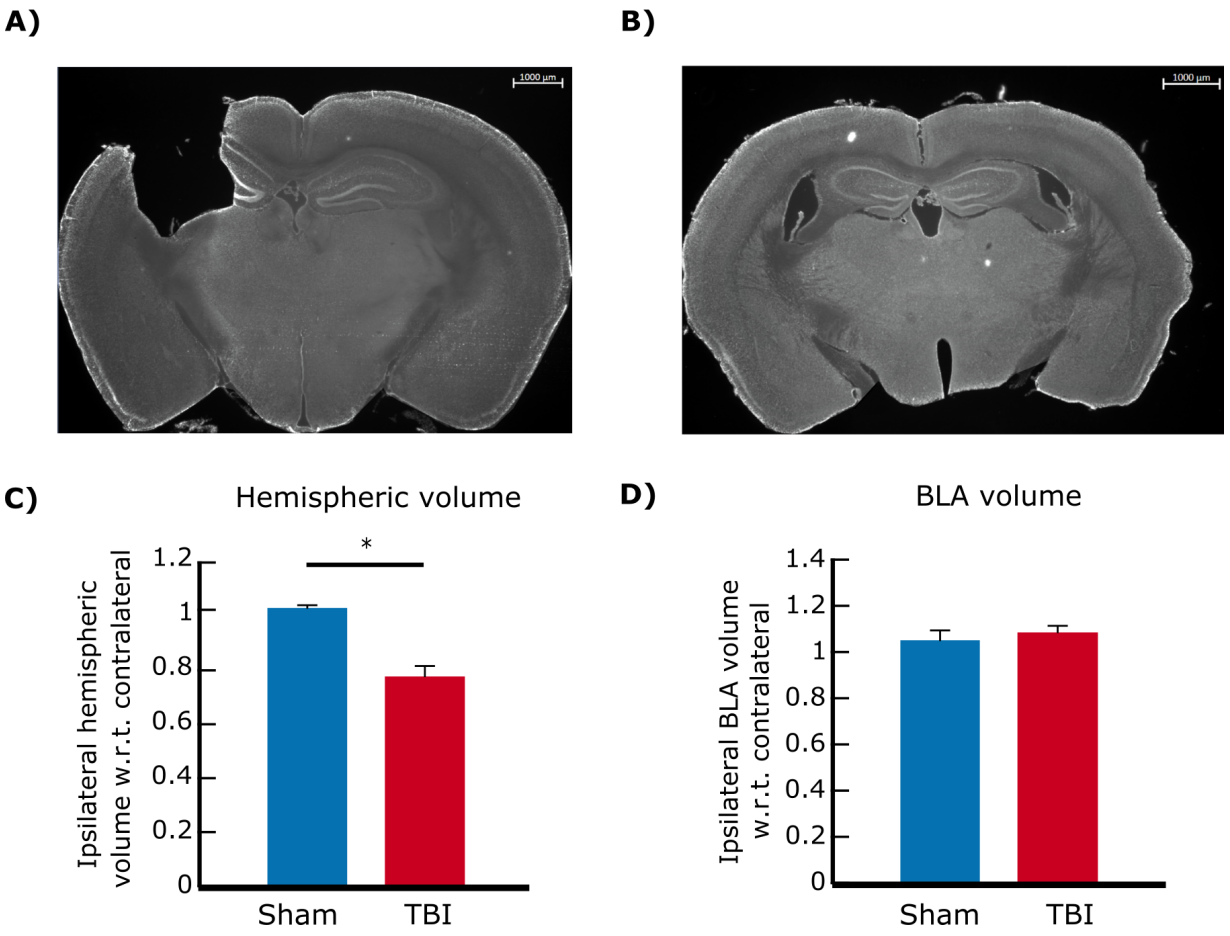


Figure 3.3. Controlled cortical impact (CCI) causes consistent injury across animals and no volumetric change in the basolateral amygdala (BLA). **(A)** 12× panoramic coronal sections stained with Fluoro Nissl, representing the lesion in the cortex and hippocampus for TBI animals and **(B)** intact areas in sham controls. **(C)** Hemispheric volume in TBI and sham animals. There is a significant reduction in the ipsilateral hemisphere volume of injured animals, compared to sham controls (*t*-test, $p < 0.05$). **(D)** Volumetric measure of the BLA. Injury does not cause volumetric change in the BLA of TBI animals. Bar graphs in **(C,D)** show mean ± SEM of data from $n = 10$ mice in the TBI condition and $n = 10$ mice in the sham condition after removal of outliers ("Materials and Methods" section); the number of outliers did not exceed one mouse for any condition; *$p < 0.05$.

control of emotional responses. To test this hypothesis, we measured volumetric changes in the BLA on week 9.

Volume of the BLA was estimated by measuring area of four sections on each side per mouse, and multiplying the area of the BLA by the distance between sections (300 μm). BLA sections were located between −1.22 and −2.18 mm Bregma. The ipsilateral volume was then normalized to its contralateral side (n = 10 for each group). There is no effect of injury on the contralateral side (data not shown). There was no significant difference in BLA volume between injured and sham mice, as shown in Figure 3.3D (two-tailed t-test, p = 0.7). These results indicate that the changes in anxiety-like behaviors were not correlated with changes in volume in the BLA.

2.4 Neural Marker: Immunostaining Indicates Upregulation of GAD Ipsilaterally

Next, we assessed functional neural correlates of the long-term affective outcomes of injury. To investigate whether the observed late decrease in anxiety had a molecular correlate, we examined the strength of inhibitory signaling in the BLA. To this end, we performed GAD immunostaining by targeting GAD65/67, which are two enzymes expressed in the brain and involved in the synthesis of GABA. GAD67 is expressed equally through the cell body, while GAD65 is mainly found in nerve terminals [206]. We chose to target GAD because it has been demonstrated that GABA plays a crucial role in anxiety disorders [157]. We quantified several metrics of GAD expression, such as number and intensity of GAD puncta. Ipsilateral values were normalized to contralateral for each mouse.

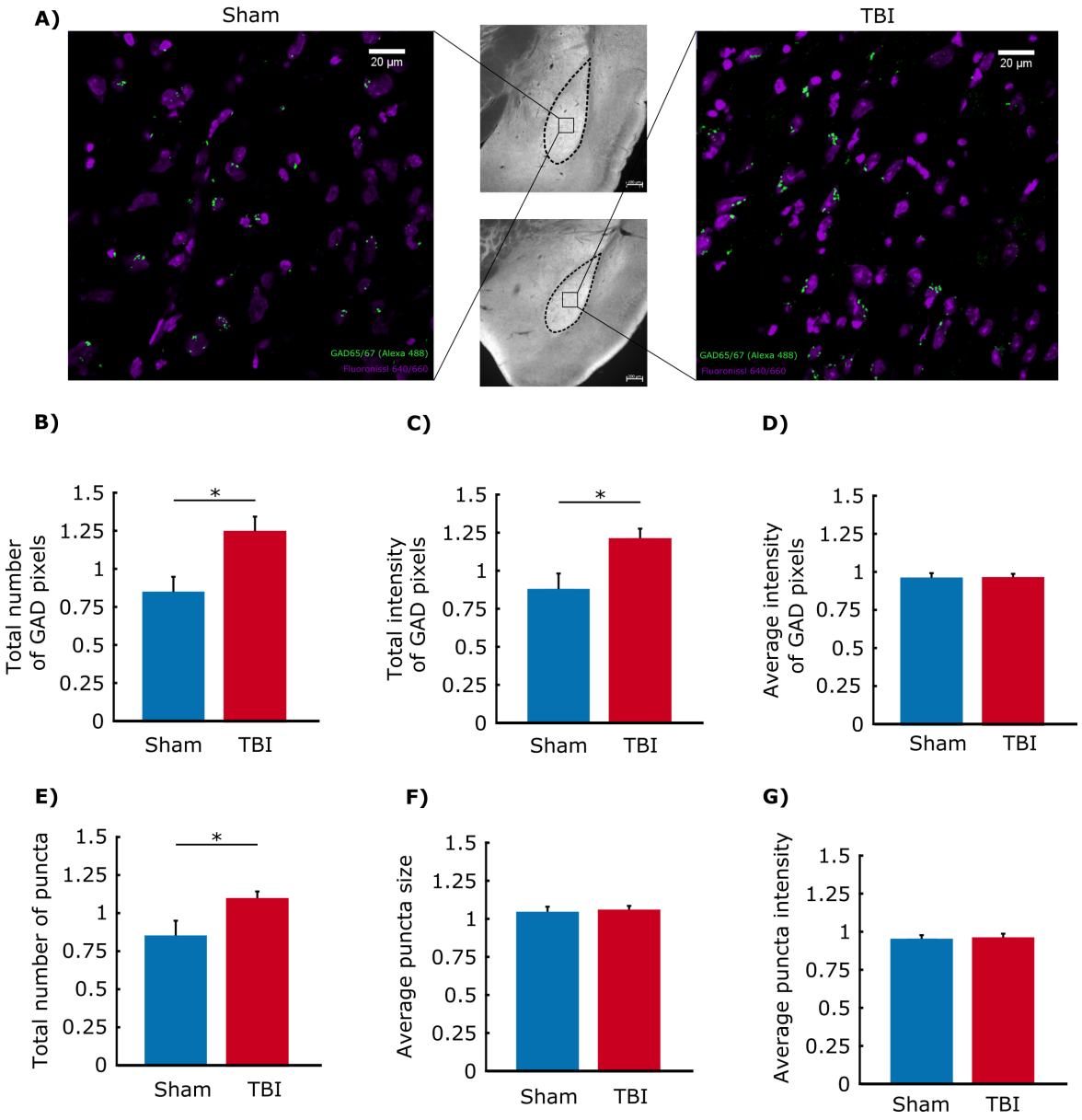


Figure 3.4. TBI is associated with upregulation of GAD immunostaining in the ipsilateral amygdala. **(A)** Middle column: 10× images of histological sections from a sham animal (top) and TBI animal (bottom). Left/Right columns: 63× views of the indicated portions of the sections. Fluoro Nissl staining in purple and GAD65/67 staining in green. **(B–G)** Quantification of GAD65/67 expression in the ipsilateral and contralateral BLA in four sections per animal. Metrics in **(B–G)** are reported as ratios of ipsilateral to contralateral values for each animal. In injured animals, there was a significant increase in the total number of GAD pixels **(B)** and the total intensity of GAD pixels **(C)**, but not the average intensity of GAD pixels **(D)**. In addition, there was an increase in the total number of GAD "puncta" (clusters of contiguous GAD pixels **(E)**, but not in the average size of puncta **(F)** or average intensity of puncta **(G)**. All bar graphs show mean ± SEM of data from $n = 10$ mice in the TBI condition and $n = 10$ mice in the sham condition after removal of outliers ("Materials and Methods" section); the number of outliers did not exceed two mice for any; *$p < 0.05$ (two-tailed t-test).

Figure 3.4A shows coronal sections zoomed-in on the BLA, with labeling of cell bodies and GAD puncta for a sham and a TBI mouse. There was an overall increase in the total number of GAD-stained pixels in the ipsilateral BLA of injured mice, measured by a two-tailed *t*-test ($p = 0.03$, Figure 3.4B. There was also an overall increase in total GAD signal intensity ($p = 0.01$, Figure 3.4C). Notably, the average intensity of GAD-stained pixels was not different between groups ($p > 0.05$, Figure 3.4D). Together, these results indicate that injury causes an increase in the number of GAD-stained pixels, but not in the intensity (brightness) of individual pixels.

To understand if there were effects on spatial clustering of GAD-stained pixels, we next analyzed the properties of groups of contiguous (or connected) GAD-pixels, called "puncta" (see "Materials and Methods" section). Compared to individual pixels, which can be contaminated by noise, puncta are more likely to represent functional signal. We found that there was an increase in the number of GAD puncta following injury (Figure 3.4E, $p = 0.04$), but no change in the average size of puncta or the average intensity of puncta ($p > 0.05$, Figures 3.4F,G). In other words, consistent with the results from individual pixels, GAD puncta are not larger or brighter, they are greater in number following injury. Together, the immunostaining results show an upregulation of GAD immunostaining in the ipsilateral BLA of TBI mice.

3. Conclusion

Human TBI has a complex pathology, and studies show that, among the many outcomes of injury, patients are at a higher risk of suffering from anxiety disorders [6, 7, 201]. Reports on the prevalence of anxiety following injury are variable: pooled long-term prevalence is reported at 36%, according to a recent review [6], but some studies suggest prevalence between 11% and

70% [7]. One unsolved issue is our lack of understanding about the neural mechanisms of injury that may increase a patient's chance of developing an anxiety disorder. Animal models of TBI are valuable to address this problem, for their ability to control for injury parameters and to allow us to measure behavioral changes and neural markers in well-controlled experiments. However, a complex, and at times contradictory, picture has emerged from various animal studies. It is important to comprehensively study these models over long time frames, to develop an understanding of TBI pathophysiology and its impact on affective behavior.

In this study, we adopt a well-established and highly controlled mouse injury model (CCI), and test mice in three assays of innate anxiety over a 7-week time-course, to evaluate how the evolving consequences of TBI impacts affective behavioral function. We demonstrate that the effects of moderate to severe TBI on anxiety-like behaviors are complex and long lasting. Additionally, with this behavioral characterization as a basis, we also measured a molecular marker of GABAergic function in the BLA, one of the key hubs of anxiety processing, and we demonstrated that there is an upregulation of GAD staining at 9 weeks post-injury.

Early after TBI, injury caused a significant increase in anxiety-like behavior measured in the EPM and EZM tests, consistent with several studies that show increased anxiety acutely after injury to the murine brain [12, 125, 141]. However, no such effect was observed in the OFT. This is consistent with Sierra-Mercado et al. (2015), who tested the effect of CCI in mice 1 week after injury, and found no change in anxiety as measured by the OFT. Interestingly, a few other studies are able to measure this early increase in anxiety in the OFT as well [12, 108, 125]. This inconsistency could be explained by differences in severity of injury [125] or rodent model [12, 108]. However, the difference between effects observed among behavioral tests in our study—

within the same group of mice at the same time points—underlines the complexity of measuring anxiety-like behavior in animal models. An important potential implication of this finding is that different assays of anxiety do not always measure the same aspect of anxiety-like behaviors. A comprehensive approach to behavioral testing following TBI is therefore imperative to draw useful conclusions. In order to study the progression of the anxiety response following injury, it is necessary to employ a repeated testing model. One concern with repeated measurement for behavioral tests of anxiety is that of potential habituation and learning. While repeated testing has been shown to have effects on anxiety tests in many studies [64, 207, 208], Bertoglio and Carobrez (2000) suggest that the decreased exploration of open arm in the EPM could be related to a qualitative shift from unconditioned to a learned form of fear response[205]. Thus, what is generally considered habituation or learning in the testing arena, is likely a change in the acquired fear response underlying the expression of anxiety-like behavior. Changes in the sham group over time suggest that some of these factors do play a role in this study. Sham mice presented habituation to the OFT and EPM, observed in the decrease in proportion of time spent in the anxiogenic zone over time post-surgery on the OF and EPM, as well as in decrease over time in the total distance traveled in the OFT. These results are consistent with previous literature, which has demonstrated that repeated exposure to the OFT and EZM lead to adaptation to the apparatus measured by decreased exploration of the anxiogenic zone and decreased overall locomotion [209-212].

While this study does not rule out these factors in the testing arena, that effect is consistent between both sham and injured groups, and differences observed between groups can be attributed to the TBI. Changes in the expression of anxiety behavior can therefore be

interpreted as a deficit in the learned fear response due to re-exposure, a deficit in learning about the context of the anxiogenic zone, or as an increase in risk taking behavior. Direct assays measuring changes in fear learning, such as acoustic startle, might help to parse out these changes over time.

The affective response to TBI is not limited to the early time points, but evolves over time, reflecting the fact that neural mechanisms of injury evolve over time [18]. Five weeks after TBI, injured mice show significantly less anxiety-like behaviors. At this stage, the mice showed an increase in exploration of the open arm in the EZM test, as compared to sham controls. The mice also display an increased tendency to explore the center anxiogenic zone in the OFT. Curiously, a similar increase in exploration of the open arm is not observed in the EPM test. Our findings are consistent with findings of decreased anxiety-like behavior in the EZM test at later time points after injury [73, 104], as well as decreased anxiety in the OFT in rats exposed to lateral fluid percussion at 1 and 3 months post-injury [73]. Interestingly, several studies have found decreased anxiety in the EPM as well, tested in mice following CCI at 20 days [10] and rats tested in a closed-head model at between 12 and 30 days [82, 83]. This difference could also be explained by the fact that we use repeated measurements of behavior in the EPM. Similarly, Ajao et al. (2012) measured an initial increase in anxiety like behavior after juvenile rats were exposed to CCI, which appears to reverse at later time points[9].

Injury also caused increased locomotion in the OFT throughout all time-points, consistent with other studies [73, 82, 125]. This suggests that the observed hyperactivity is independent of the early increase and later decrease in anxiety-like behavior. These findings are consistent with

human studies where hyperactivity has been reported following pediatric TBI [213] and impulsivity has been reported following TBI in adults [120, 214].

Several neural mechanisms underlying anxiety changes following injury have been investigated [9, 12, 14, 70, 80, 83, 134, 141]. Of particular interest is the BLA, which has been linked to changes in anxiety-like behaviors. Causal manipulations of projections from and to the BLA directly alter anxiety-like behaviors [215-219], indicating that this area is an important hub for emotional responses in the brain. In addition, signaling by GABA, the major inhibitory neurotransmitter in the nervous system, is greatly implicated in psychiatric disorders such as anxiety and depression [156, 157, 159, 220]. Infusion of GABAA receptor antagonist into the amygdala increases anxiety [221], whereas GABA agonist infusion into the BLA reduces anxiety [222], indicating that the inhibitory balance within the amygdala drives changes in anxiety. In previous TBI studies, it has been shown that reduced GABAergic inhibition and increase in number of neurons in the BLA correlate with enhanced anxiety in rodents early after injury [12, 141].

In this study, while the volumetric measures of the peri-injury area indicate a reduction in ipsilateral hemispheric volume as expected [10, 12, 222], there is no significant difference in volumetric measures of the BLA between injured mice and sham controls. However, GAD immunostaining is upregulated in the ipsilateral BLA at 9 weeks following injury, indicating increased GABAergic inhibition within this area. This increase is correlated with late decreases in anxiety-like behavior of the injured animals at later time points. Our results are consistent with causal, GABAA agonist infusion experiments in the BLA [47]. Almeida-Suhett et al. (2014) observed TBI-induced decrease in GABAergic inhibition and an increase in anxiety-like

behavior at early time points after injury, whereas this study shows the opposite effects at later time points. Thus, our results regarding GABA signaling are in line with previous work and extend our understanding about the longer-term effects of injury.

In summary, we demonstrate a time-dependent reversal in the course of affective behavioral response following traumatic injury to the mouse brain: an early increase followed by a late decrease in anxiety, with the latter being correlated with an increase in GABAergic inhibition in the BLA. In addition to revealing a complex affective trajectory, results support the hypothesis that the lack of consensus across past studies [10, 73, 82] on the effects on anxiety outcomes following injury may be the result of the variability in injury models used, behavioral assays of anxiety chosen and time-points at which assessments were made. Consequently, they highlight the need for the use of a reproducible model of injury as well as the use of multiple assays and time-points within future studies. Such an approach can provide a consistent foundation for investigating the neural mechanisms underlying affective outcomes of TBI and the development of therapeutic strategies.

Chapter 4: Resilience and vulnerability in anxiety outcomes of TBI

1. Introduction

Neuropsychiatric disorders are a significant outcome of traumatic brain injury (TBI), and may develop months to years after the incident [7, 174]. Among the neuropsychiatric outcomes of TBI, anxiety-related disorders are highly prevalent and impose significant impairments to patients. Some studies report the prevalence of anxiety-related disorders after TBI ranges between 10% to 70% [223], and a recent meta-analysis suggests that the pooled prevalence of anxiety following TBI is 20% in the first year, and 36% five years post-injury [6]. Those results indicate that, although the prevalence of anxiety disorders post-TBI is high, only a sub-set of TBI patients will be affected. Therefore, clinical studies aiming to address anxiety outcomes of TBI adopt rigorous exclusion criteria, such as structured interviews and scales, to determine which patients should be included in studies [224, 225]. On the other hand, pre-clinical studies of TBI and anxiety-like behaviors have generally included the entire injured population as a homogenous group, and the physiological or molecular sequelae discussed as a function of the injury.

A variety of effects has been reported in anxiety-like behaviors in these studies, suggesting that heterogenous responses within the injured group may be a factor [10, 12, 14, 73, 82, 88, 141]. Here, we aim to address this issue by developing an approach analogous to the use of inclusion criteria in clinical studies. In a mouse model of controlled cortical impact injury, we measure a multidimensional behavioral profile which is then clustered and validated to distinguish between sub-groups of injured animals. Our results suggest that this alternative, data-driven approach to analyzing dysfunction in anxiety-like behaviors following traumatic brain

injury, allows us to distinguish subjects that present maladaptative anxiety after the injury from those that are unaffected by the injury, and that we can subsequently measure distinctions in molecular markers between these sub-groups.

A number of pre-clinical studies have addressed the impact of brain injury on changes in anxiety-like behavioral responses. These studies report a large range of effects, from decreased anxiety following a controlled cortical impact injury [10] and weight-drop injury [82] at 20 to 30 days post-TBI; increase in anxiety following weight-drop [141], lateral fluid percussion [13], and blast overpressure [79], up to 3 months after later; and no change on anxiety on animals exposed to a CCI [89], repeated weight-drop [90] and LFP [96], up to 3 months after injury. We have previously demonstrated this complexity in observed anxiety outcomes, demonstrating a time and task-dependent trajectory in response [117]. We found that the TBI group demonstrates an increase in anxiety-like behavior early after injury, followed by a decrease at later time-points [117]. However, consistent with previous approaches to analyzing behavioral dysfunction, this study compares injured and sham groups, disregarding any potential variability in injury response within the injured group.

We began to question whether the approach of treating the injury group as one, obscures the effect of heterogeneity in behavioral response following injury. Such distinctions in response to a common stimulus have been described in studies analyzing the effects of psychological stress [175, 177, 181]. The notion of individual differences in response to a stressor has been defined in the neurobiological and psychological literature as resilience [226, 227], or the organism's ability to adapt to adversity. Resilient individuals cope better with stressful events, being able to adapt to extreme situations, whereas vulnerable individuals present higher risk of

some types of psychiatric disorders, in particular anxiety and depression [228]. Thus, maladapted or well-adapted subjects can also be described as having different levels of resilience to the stressor, which leads individuals exposed to extreme situations to present potentially opposing behavioral outcomes following exposure to the same stressor [227].

Several insights about the underlying mechanisms leading to resilience and vulnerability to psychological stressors have been identified using approaches leveraging heterogeneity. To identify these sub-groups, arbitrarily selected severity measures, the cut-off behavioral criteria (CBC), were used to distinguish subjects that are well-adapted or maladapted in their response to a common stressor [175]. The observed response in the elevated plus maze and in the acoustic startle response determine if they present a maladapted or well-adapted behavior profile. Animals who present high avoidance to the open arm, and increased startle response are deemed maladapted, or low-responders, whereas animals who present no change compared to controls are considered well-adapted, or high-responders. These behaviorally defined sub-groups, who were subjected to an identical stressor, show distinctions in physiological measures as increased plasma corticosterone and ACTH levels, increased sympathetic activity, diminished vagal tone and increased sympathovagal balance [96].

The prevalence of extreme (maladapted) responders across different strain of rats varied, and reductions were associated with a blunted hypothalamic pituitary axis response to stress [178]. Early post-stressor intervention with a selective serotonin reuptake inhibitor was found to reduce incidence of extreme behavioral responses [229]. Early-life exposure to stressors led to an increased vulnerability to developing a maladapted phenotype and persisting physiological

abnormalities later in life, and was associated with a failure of recovery from the initial autonomic nervous system response to stress exposure [182].

Such research supports the development of an approach that defines behavioral criteria addressing the variance in individual response pattern and magnitude, following the exposure to traumatic injury. Classification of animals exposed to injury into definable groups, presenting vulnerability or resilience in their behavioral responses, allows for focused study that incorporates the underlying heterogeneity. Based on our previous findings [117], several different assays and metrics of anxiety are measured over time to give rise to a multi-dimensional behavioral profile. We therefore need to use principal component analysis followed by clustering to identify unique sub-sets within this multi-dimensional dataset. This is followed by validation of the distinctness of these clusters by plotting the individual behavioral metrics for each separately. Identification of behaviorally distinct sub-sets allows us to then further probe distinctions in molecular signaling in several brain regions known to be associated with anxiety-like behavior, such as the medial prefrontal cortex (mPFC), the basolateral amygdala (BLA), and the ventral hippocampus (vHPC) [218, 230-232]. Activation of ventromedial prefrontal cortex-amygdala (vmPFC-Amy) pathway and amygdala reactivity have been associated with behavioral adaptation and vulnerability to neuropsychiatric disorders [218, 231]. Modulation of GABA and glutamate activity in various limbic areas is associated with anxiety-like behaviors [157, 165, 206, 233-237]. On the other hand, neuropeptide Y has been identified as a marker of resilience in behavioral outcomes to stress exposure [181, 238, 239]. We characterize the molecular signatures of signaling via GABA (GAD65/67), glutamate (vGLUT), and neuropeptide Y (NPY), which are all known to be involved in changes in signaling in anxiety response or resilience [181,

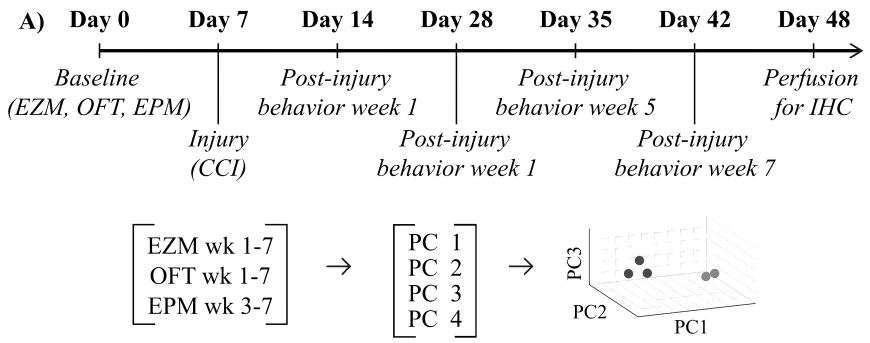


Figure 4.1: Approach for dividing tbi animals into two groups based on multidimensional behavioral profile A) Top: Schematics of experimental timeline, including baseline behavioral testing, injury, post-injury behavioral testing and immunostaining procedures. Bottom: schematics of behavioral matrix used as input for PCA analysis. Behavioral data on the EZM, OFT and EPM were combined to generate a behavioral profile for each animal.

238-240]. Our results suggest that behaviorally identified resilient and vulnerable sub-groups following injury show distinct changes in these molecular markers. Moreover, there is a significant correlation of the molecular change with the extent of behavioral change in the vulnerable group.

2. Results

Mice were first tested on three commonly used behavioral assays for anxiety, namely, the EZM, OFT and EPM [62, 107, 207]. Metrics of anxiety-like behaviors as well as of general locomotion were measured before injury (Fig. 4.1A), and were denoted as 'baseline' measurements. A week following the baseline measurements, animals underwent either sham surgery ('control' group) or CCI injury ('TBI' group); mice were assigned randomly to the two groups. After a week of recovery, all animals were tested on the three behavioral assays, once every two weeks for a period of seven weeks following injury, resulting in four measurement time-points post-surgery (sham or TBI). Mice were sacrificed at 8 weeks after injury. In previous work [117], we have shown that effects of injury on anxiety-like behaviors are time- and task-dependent, and follow a complex trajectory. Additionally, while habituation to the testing arena was observed in OFT and EPM, it was consistent across sham and injury groups. However, this approach does not allow us to distinguish if the individual response to injury varied between animals in the TBI group. In this study, we use a multi-dimensional clustering approach to address this question. For each animal, behavioral measurements were normalized to their respective baseline values to account for pre-surgery variability across animals.

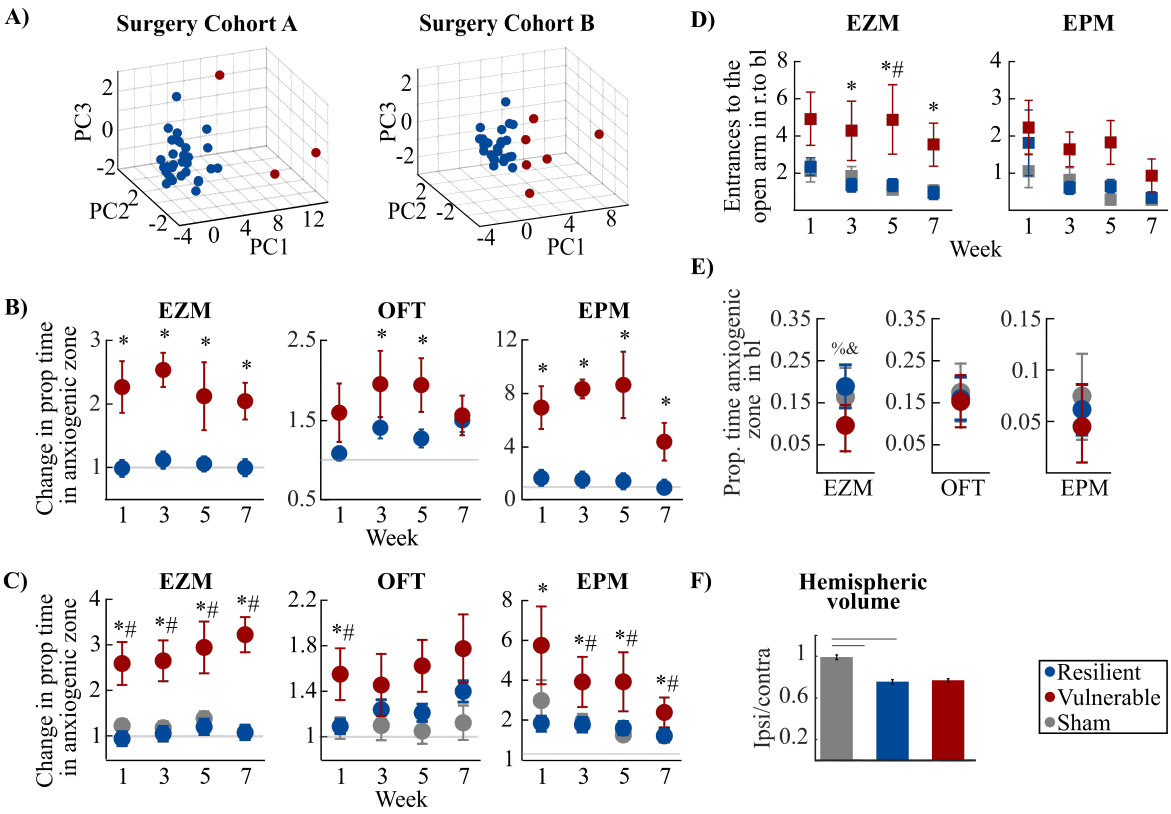


Figure 4.2: TBI animals present distinct behavioral profiles, indicating different levels of vulnerability to anxiety following TBI. A) First three principal components on surgery cohort A and B, showing TBI animals separated into two clusters. B) Proportion of time in the anxiogenic zone for surgery cohort A. In the EZM (left), there was a main effect of treatment ($F=19.89$, $p=0$), and vulnerable animals present decreased anxiety compared to resilient animals on all weeks (*: $p<0.05$). In the OFT (center), there was a main effect of treatment ($F=10.06$ $p=0$), and anxiety was decreased for vulnerable animals compared to resilient animals on weeks three and five ($p<0.05$). In the EPM (right), there was a main effect of treatment ($F=120.07$, $p=0$), and vulnerable animals presented decreased anxiety compared to resilient animals on all time-points ($p<0.05$). C) Proportion of time in the open arm in the anxiogenic zone, combining surgery cohorts A and B. In the EZM, (left), there was a main effect of treatment ($F=95$, $p=0$), and vulnerable animals had decreased anxiety compared to shams and resilient animals in all time-points (* and #: $p<0.05$). In the OFT (center) there was a main effect of treatment ($F=8.37$, $p=0.0003$), and vulnerable animals presented decreased anxiety on week one ($p<0.05$). In the EPM (right), there was a main effect of treatment ($F=12.45$, $p=0$), and vulnerable animals were significantly less anxious compared to shams on weeks three, five and seven and to resilient animals on all time-points ($p<0.05$). D) Number of entrances to the open arm in the EZM (left) and EPM (right), combining cohorts A and B. There was a main effect of treatment in the EZM ($F=3.92$, $p=0.02$), and vulnerable animals had more entrances than shams on week five, and more entrances than resilient animals on weeks three, five and seven ($p<0.05$), and no effect in the EPM $p=0.18$, $F(2)=1.68$. E) Proportion of time in the anxiogenic zone in the anxiogenic zone during baseline. There was a main effect of treatment in the EZM (left, $F=4.97$, $p=0.008$), with vulnerable animals showing increased anxiety compared to sham and resilient animals ($p<0.05$), but there was no effect of treatment on the OFT (center) and EPM (right). F) Ipsilateral hemispheric volume in comparison to contralateral side. There was a significant reduction in volumetric hemisphere for both resilient and vulnerable animals, in comparison to sham animals ($p<0.001$), however, resilient and vulnerable animals did not differ from each other ($p=0.67$). Lines represent $p<0.05$ for difference between groups with post-hoc t-test with FDR correction. Bars represent SEM, * represents $p<0.05$ for vulnerable versus resilient, # represents $p<0.05$ for vulnerable versus sham after post-hoc t-test with false discovery rate (FDR) correction. All data presented includes outliers. Behavioral data is normalized to each individual animal's baseline. Data from 103 animals (sham: n=32, resilient: n=62, vulnerable: n=9)

2.1 Identification of two behaviorally distinct sub-groups (vulnerable and resilient) following TBI, based on a multidimensional behavioral profile of each individual.

The proportion of time that mice spend in the exposed zones of behavioral arenas – open arms of the EZM and EPM, and the central zone of the OFT – is a standard metric used to quantify their anxiety-like behaviors [14, 62, 101, 107, 207]. We refer to it generally as 'proportion of time spent in the anxiogenic zone'. Our post-injury measurements (baseline-normalized) of this metric across mazes and time points yielded a 11-dimensional behavioral vector or 'profile' over time for each mouse. We asked if, based on their multidimensional behavioral profiles, we could identify distinct groups within the TBI cohort: one exhibiting substantial affective behavioral consequences of injury, and another being largely resistant to effects of injury. To address this question, we developed an unbiased data-driven approach.

We obtained the 11-dimensional behavioral profile for Cohort A of 37 TBI animals (and 15 sham animals; animals assigned to conditions randomly), and organized this data into 37x11 behavioral profile matrix (for TBI animals, and 15x11 matrix for shams). We started the analysis by performing principal components analysis on this multidimensional dataset for TBI animals (in this case, 37 x 11) to determine the smallest number of behavioral dimensions that accounted for most of the variability in the data. We find that 92% of the variance was explained by just 4 of the 11 dimensions (Fig. 4.2A). Next, we applied k-means clustering to the lower dimensional dataset (of principal components; 37x4) to force the identification of two distinct clusters. Since PCA transforms data points into a new coordinate frame, it was not possible, a priori, to determine whether the clusters in PCA space represented low- and high-responders, and if so, which cluster represented what kind of response.

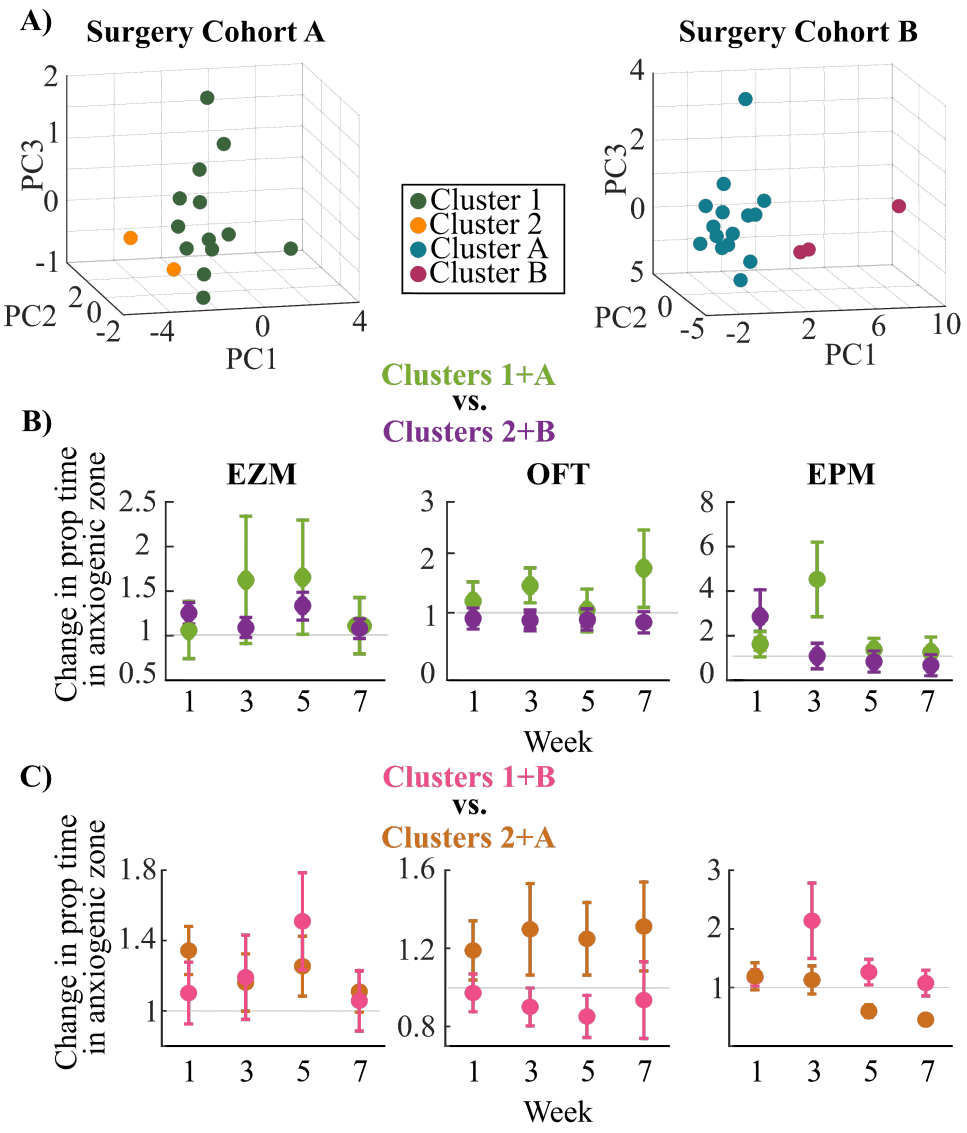


Figure 4.3: Clustering sham animals does not produce behaviorally distinct groups. A) PCA done independently on sham animals, for surgery cohort A and B separately. Animals were then clustered into two separated groups by k-means, similarly to the procedure applied for TBI animals. B) Proportion of time in the anxiogenic zones comparing cluster 1+A versus cluster 2+B. In the EZM (left) and EPM (right) there was no main effect of treatment (EZM: F=0.95 p=0.33; EPM: F=1.14 p=0.28) In the OFT (center), there was a small effect of treatment (F=8.8 p=0.003), with no post-hoc effect. C) Proportion of time in the anxiogenic zones, combining cluster 1+B versus cluster 2+A. In the EZM (left), there was no main effect of treatment (F=0 p=0.98). In the OFT (center) and EPM (right), there was a main effect of treatment (OFT: F=8.65 p=0.003; EPM: F=7.37 p=0.007), with no post-hoc effect. Bars represent SEM. Data from 32 sham animals, including outliers. Data is normalized to each individual animal's baseline.

To determine if animals in these two clusters corresponded to distinct responses to TBI, we plotted for each assay, the values of anxiety metrics of animals in the two clusters (Fig.4.2B). We find that animals in the red cluster showed a significant difference with respect to animals from the blue cluster (Fig. 4.2B; repeated thee-way ANOVA, EZM: main effect: $F=19.89$, $p=0$; OFT: main effect: $F=10.06$ $p=0$; EPM: main effect: $F=120.07$, $p=0$). Animals in the blue cluster showed minimal change in anxiety metrics over time with respect to sham animals (Fig. 4.2B; post-hoc t-test with FDR correction, $p>0.05$). These results reveal that the two groups exhibit consistently distinct behavioral patterns across anxiety metrics and time following injury. Moreover, they demonstrate that animals in the blue cluster were largely 'resilient' to TBI (not different from sham), whereas animals in the red cluster were 'vulnerable' to injury (different from resilient and sham). We refer to this approach of identifying distinct behavioral groups from multidimensional profiles as multidimensional behavioral clustering with validation (MBCV). In this cohort, we found that only 3/37 animals were identified as vulnerable.

To increase the size of the vulnerable sample (for subsequent analyses), we repeated the experiment on a different cohort of 34 TBI animals (and 17 sham animals), and applied our MBCV analysis approach to this dataset (Fig. 4.2A; right panel). Because the two cohorts were separated by a 1.5 year window, we applied PCA and clustering independently to data from this second cohort. We identified 6 vulnerable and 28 resilient subjects in this cohort (behavior data for this cohort is shown on Sup. Figure 4.1B).

For all subsequent analyses, we merged the vulnerable (as well as resilient) groups from the two cohorts. First, we compare the proportion of time spent by animals vulnerable to TBI,

animals resilient to TBI as well as sham animals in the anxiogenic zone in each of the behavioral assays (Fig. 4.2C). We find systematic differences between the vulnerable and both resilient and sham animals, but no differences between the resilient and sham animals (Fig. 4.2C; EZM: repeated three-way ANOVA, main effect of treatment, $F=95$, $p=0$, post-hoc: vulnerable versus resilient and vulnerable versus sham, $p<0.01$ in all time-points. OFT: repeated three-way ANOVA, main effect of treatment, $F=8.37$, $p=0.0003$, post-hoc: vulnerable versus resilient and vulnerable versus sham $p<0.05$ on week one. EPM: repeated three-way ANOVA: main effect of treatment, $F=12.45$, $p=0$, effect of time, $F=4.05$, $p=0.007$, post-hoc, vulnerable versus resilient, $p<0.05$ in all time-points, vulnerable versus sham, $p<0.05$ on weeks three, five and seven).

Specifically, vulnerable animals spent a significantly higher proportion of time exploring the open arms in both the EPM and the EZM, and a trend towards greater exploration of the center in the OFT, as compared to the resilient animals (Fig. 4.2C). By contrast, in the EZM and EPM, resilient and sham animals showed no changes from baseline in the proportion of time spent in the anxiogenic zone (post-hoc t-test with FDR correction, $p>0.05$).

To further validate our approach, we investigated whether vulnerable versus resilient animals exhibited consistent results with respect to a different anxiety metric, one that was not used in the MBCV analysis to identify the groups. To this end, we compare a secondary anxiety metric between these groups of animals, namely, the number of entrances to the open arm for the EZM and the EPM (Fig. 4.2D), and find similar results to those from proportion of time spent in the anxiety zone. In the EZM we show that vulnerable animals present a greater number of entrances into the open arm than resilient and sham animals (Fig. 4.2D, left: repeated three-way ANOVA, $F=3.92$, $p=0.02$), with significant differences observed between vulnerable and resilient

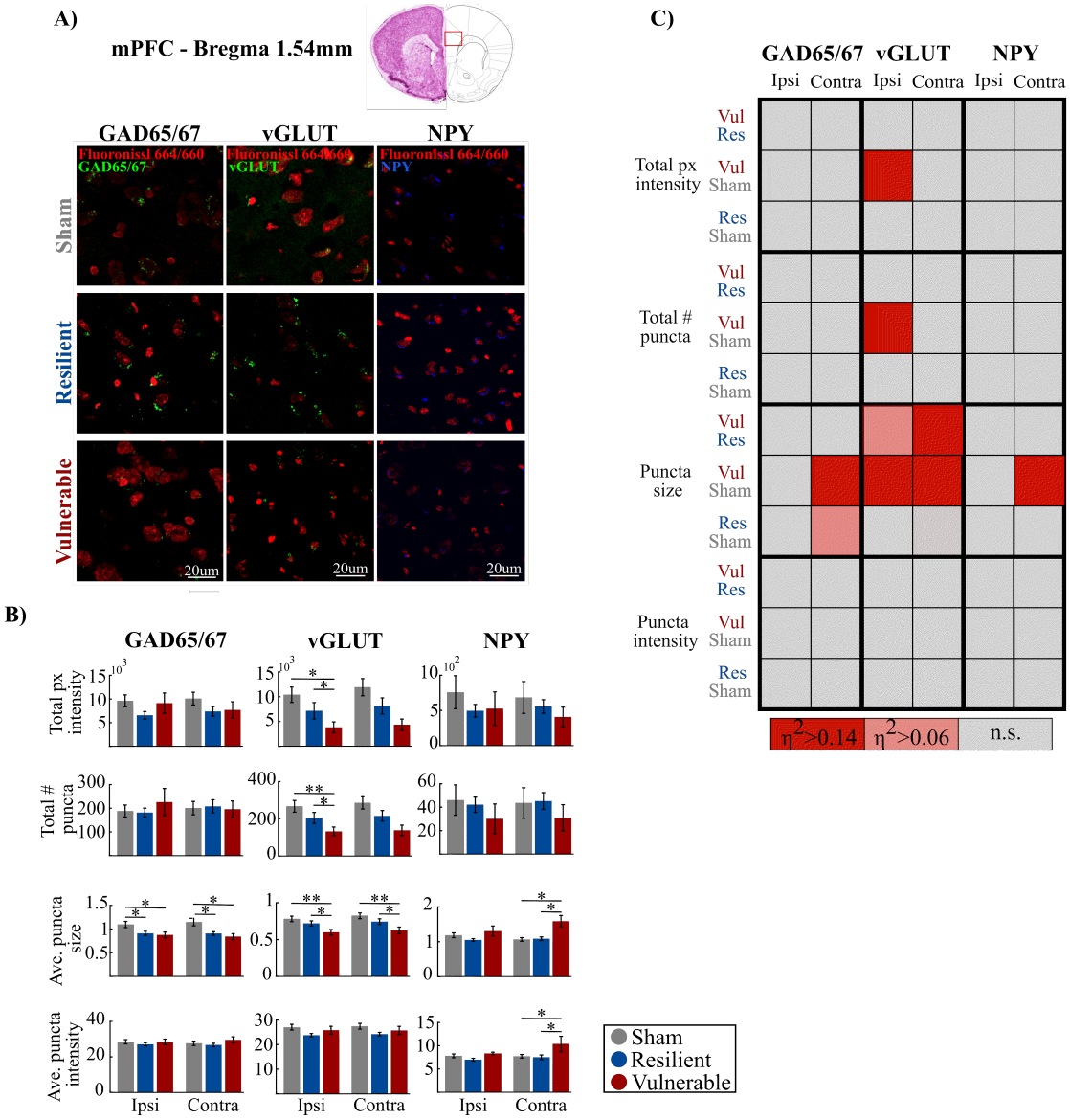


Figure 4.4: Vulnerable animals present downregulation of GAD and vGLUT, and upregulation of NPY immunostaining in the mPFC. A) Top: schematics of brain sectioning showing where mPFC images were taken. Bottom: example of GAD65/67, vGLUT and NPY staining for resilient, vulnerable and sham animals B) Eta-squared effect size in the mPFC. comparing vulnerable animals to resilient and shams, and resilient animals to shams. Colors indicate strength of effect for metrics that presented a statistically significant difference (post-hoc: $p<0.05$). C) Pixel and puncta analysis in the mPFC, for GAD65/67, vGLUT and NPY. Bars represent SEM. Data from 53 animals (vulnerable=9, resilient=28, sham=16), including outliers. *: $p<0.05$ and **: $p<0.01$, for difference between groups by post-hoc t-test with false discovery rate (FDR) correction.

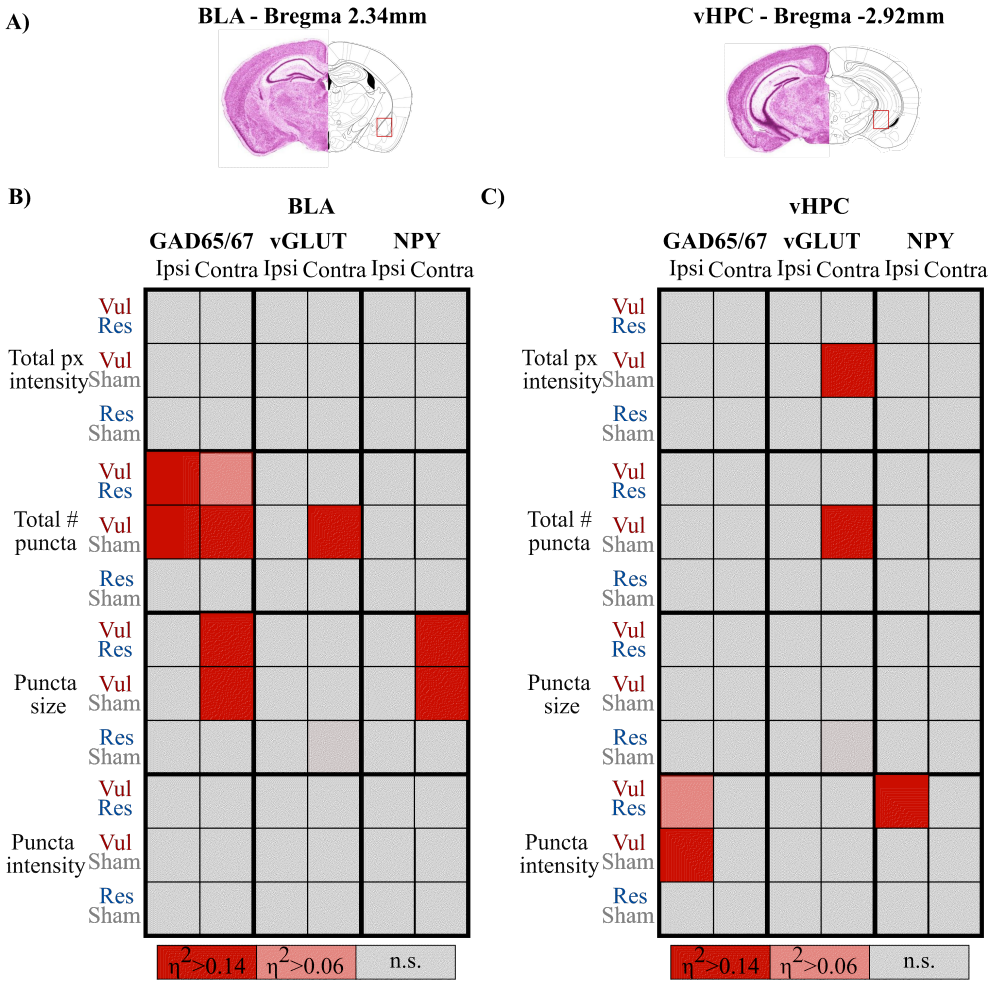


Figure 4.5: Vulnerable animals present significant differences compared to resilient and sham animals in the BLA and vHPC. A) Schematics of brain sectioning showing where BLA and vHPC images were taken. B-C) Eta-squared effect size in the BLA (B) and vHPC (C), comparing vulnerable animals to resilient and shams, and resilient animals to shams. Colors indicate strength of effect for metrics that presented a significant statistical difference. Data from 53 animals (vulnerable=9, resilient=28, sham=16), including outliers.

animals on weeks three, five and seven ($p<0.05$, post-hoc t-tests with FDR correction), and significant differences between vulnerable and sham animals on weeks five and seven ($p<0.05$, post-hoc t-tests with FDR correction). In the EPM, there is no significant difference between groups (repeated three-way ANOVA, $F=1.68$, $p=0.18$), but there was a trend indicating that vulnerable animals presented more entrances into the open arm compared to resilient and control animals. Thus, findings based on our MBCV approach generalize, and are robust to the anxiety metric used.

We next assessed potential deficits in general locomotion by measuring the total distance travelled by mice in each of the three assays (Sup. Fig 4.1C). There is no difference in locomotion (normalized to baseline) between groups in the EZM (repeated three-way ANOVA, $F=0.17$, $p=0.85$,). In the EPM, there was a main effect of injury (repeated three-way ANOVA, $F(2)=3.84$, $p=0.02$,), but no significant post-hoc effect. In the OFT, there was a main effect of injury (repeated three-way ANOVA, $F=29.74$, $p=0$,) with vulnerable (but not resilient) animals being significantly more active than sham controls on weeks one, three, five and seven ($p<0.05$, post-hoc t-tests with FDR correction). These results are in agreement with previous findings that suggest increased overall locomotor activity following injury in the open field test. Notably, however, locomotion is not a confounding factor for the observed differences in anxiety-like behavior, between the vulnerable and resilient groups, in the EZM or the EPM.

We wondered whether the resilient vs. vulnerable grouping was already evident in baseline measurements. We compared the anxiety metrics prior to injury for animals from these groups, and found no systematic differences in any of the anxiety tests (Fig. 4.2E). The

vulnerable group does not show differences from either the resilient or sham groups for either the EPM or the OFT (three-way ANOVA; OFT: $F=1.01$, $p=0.36$,; EPM: $F=1.81$, $p=0.16$,). Interestingly, in the EZM, vulnerable animals spent less time in the open arm during baseline, compared to the resilient and sham animals (Fig 4.2E-left, EZM: $F=4.97$, $p=0.008$). These results show that the enhanced exploratory behavior in the vulnerable group, compared to the resilient (and sham) group, arises subsequent to the injury.

We also wondered whether differences in injury severity between groups might account for their anxiety phenotypes. To this end, for each animal, we assessed the volume of the injured hemisphere (ipsi) relative to the volume of the uninjured hemisphere (contra) and compared across groups (Fig. 4.2F). We find a reduction in the normalized (ipsi/contra) hemispheric volume in the peri-injury area in both the TBI groups (vulnerable and resilient), compared to sham controls (three-way ANOVA, $F=39.63$, $p<0.001$, effect of treatment; $p<0.001$, post-hoc t-test with FDR correction, vulnerable and resilient vs. sham). However, there was no difference between vulnerable and resilient animals (t-test, $p=0.67$). Additionally, there was no effect of the contralateral peri-injury hemispheric volume in TBI animals compared to sham controls (Supplementary Table 4, three-way ANOVA, $F=1.52$, $p=0.23$).

Our approach to identify distinct sub-groups within TBI animals was motivated by the hypothesis that the distributions of behavioral measures following injury versus sham surgery may overlap significantly, thereby making it difficult to isolate reliable differences in behavioral outcomes (and neural mechanisms) by the standard approach of comparing between these groups. To test if this underlying hypothesis was true, we plotted the distributions of behavioral

metrics in the TBI and sham groups. We found that this is, in fact, true: the distributions of anxiety behavioral metrics in the TBI and sham control groups exhibit substantial overlap (Sup. Fig. 4.1A).

Taken together, the above results establish the MBCV as a reliable approach for identifying two groups of animals, vulnerable and resilient, with distinct anxiety-like behavioral outcomes following injury.

2.2 Application of MBCV approach to sham animals does not yield behaviorally distinct sub-groups.

The success of MBCV approach applied to the TBI cohorts raises the question as to whether this approach is effective specifically for the TBI cohorts, or whether the fundamental idea of identifying two groups, applied to any distribution (batch of animals) can yield groups with distinct behavioral (specifically, anxiety) signatures due to intrinsic variability within distributions. Since each cohort also had, associated with it, a sham surgery group where the same behavioral measures were collected, we decided to test this question specifically on the sham animals. We applied PCA and clustering to each sham cohort, independently (Fig 4.3A). We then examined the individual behavioral metrics for the two clusters in each cohort, and found no systematic differences (Sup. Fig. 4.3). For improved statistical power for this comparison, we combined clusters across cohorts in both possible ways and found no systematic differences between the resulting groups, for either case (Fig. 4.3B: EZM, $F=0.95$ $p=0.33$; EPM, $F=1.14$ $p=0.28$; Fig. 4.3C: EZM, $p=0.33$, $F(2)=0.95$; OFT, $p=0.003$, $F=8.65$, orange animals

show higher values than pink animals; EPM, p=0.007, F=7.37, orange animals show lower values than pink animals, no post hoc effect). In other words, MBCV applied to sham animals did not produce groups that exhibited distinct patterns of behavioral outcomes that were consistent across anxiety metrics.

2.3 Differences in mPFC molecular markers between vulnerable and resilient groups

Having identified vulnerable versus resilient groups based on their anxiety behavioral profiles, we next asked if there were systematic molecular differences that could serve as neural signatures of vulnerability and resilience. To assess molecular differences, we perfused the brains of the animals and performed immunohistochemistry. Specifically, we measured expression of three markers - an inhibitory marker (GAD65/67), an excitatory marker (vGLUT), and neuropeptide Y (NPY), in the mPFC, a key brain region involved in the control of anxiety [232]. Figures 4.4A and 4.5A show the location of imaging and sample images for all three markers in all animal groups (resilient, vulnerable and sham).

Staining for GAD in the mPFC showed a downregulation in both resilient and vulnerable animals compared to sham controls. There was a significant decrease in the average size of GAD 65/67 puncta in the ipsilateral as well as contralateral sides (Fig 4.4B- left column, third row; ANOVA, F=4.07, p=0.02; contra: ANOVA, F=6.54, p=0.003; post-hoc t-test with FDR correction, $p<0.05$ for both sides of the brain). However, there were no differences in GAD staining between vulnerable and resilient animals.

Figure 6

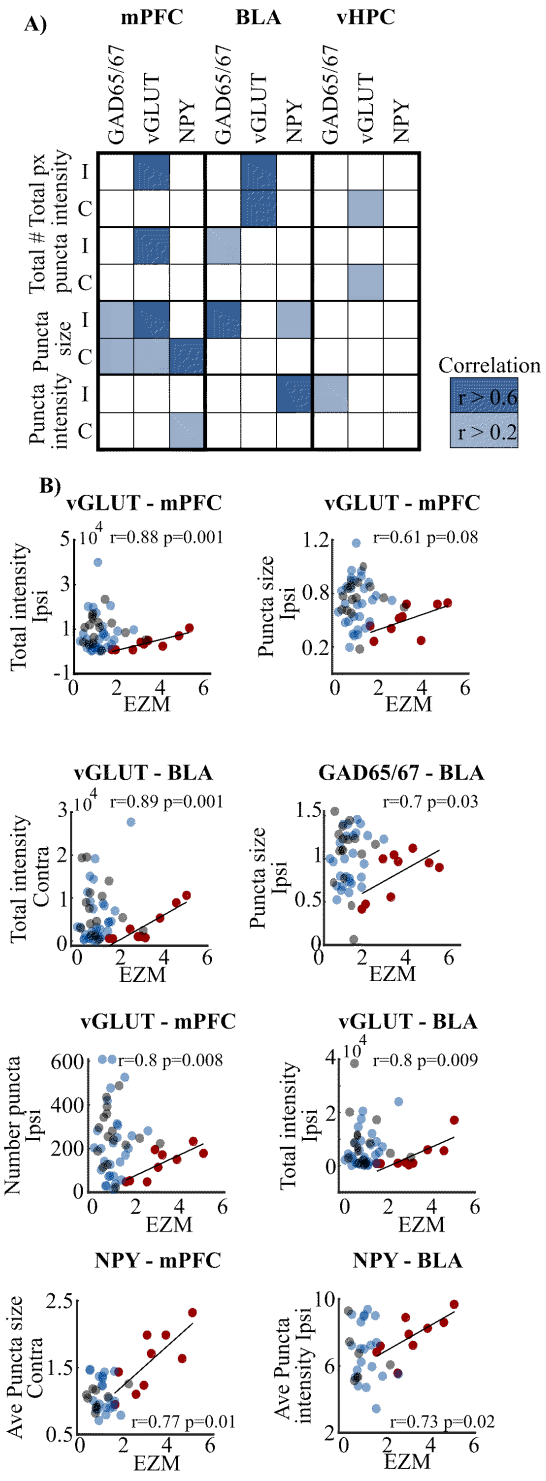


Figure 4.6: molecular changes in vulnerable animals correlate with behavioral outcomes in the EZM on week seven. A) Correlation values between molecular metrics and behavioral results for vulnerable animals in the EZM on week seven. Colors indicate strength of correlation, whenever there was a significant main effect in the molecular metric. B) Correlation plots showing strong correlations between molecular metric and behavior. Data from 9 vulnerable animals, no outliers were removed.

Staining for vGLUT in the mPFC showed a downregulation specifically in vulnerable animals compared to sham controls. The total intensity of vGLUT pixels was marginally lower, and number of their puncta and the average puncta size were significantly lower on the ipsilateral side in vulnerable animals (Fig. 4.4B-middle column, top three rows; pixel intensity: ANOVA, $F=2.31$, $p=0.1$; puncta number: ANOVA, $F=3.35$, $p=0.04$, post-hoc t-test with FDR correction $p<0.05$; puncta size: ANOVA: $F=3.44$, $p=0.04$, post-hoc t-test with FDR correction, $p<0.05$). The average puncta size was lower on the contralateral side as well for vulnerable animals in comparison to sham controls (Fig. 4.4B - middle column, third row; ANOVA: $F=3.4$, $p=0.04$, post-hoc t-test with FDR correction, $p<0.05$). Notably, in all cases, there were no differences between the resilient animals and sham controls.

Staining for NPY, a marker for resilience to stress [181, 240], on the other hand, showed an upregulation in the mPFC, and specifically in vulnerable animals. The average puncta size and intensity were significantly higher on the contralateral side in vulnerable animals (Fig. 4.4B - right column, bottom two rows; size: ANOVA, $F=8.99$, $p=0.0007$; intensity: $F=3.53$, $p=0.03$). Notably, in both cases, vulnerable animals were different from both control and resilient animals for the average puncta size (post-hoc t-test with FDR correction, $p<0.05$), and there were no differences between resilient and sham animals.

To visualize these effects succinctly, we plotted a matrix of effect sizes observed in the comparisons between all pairs of groups (vulnerable vs resilient, vulnerable vs sham, and resilient vs sham) for each of these molecular measures (Fig. 4.4C); the size of the effect was coded by color. This matrix indicates clearly that the resilient group does not show strong

changes with respect to control (η^2>0.06) in any of the metrics measured. By contrast, the vulnerable group shows a strong effect size (η^2>0.06) with respect to sham control in some metrics, such as puncta size of GAD 65/67 staining, puncta size, number and total pixel intensity of vGLUT immunostaining, and puncta size for NPY immunostaining (GAD: vulnerable vs sham, puncta size, contra: η^2=0.22, p=0.01; resilient vs sham, puncta size, contra: η^2=0.22, p=0.01; vGLUT: vulnerable vs sham, puncta size, ipsi: η^2=0.3, p=0.003, contra η^2=0.29, p=0.004; number of puncta, ipsi: η^2=0.27, p=0.006; total px intensity, ipsi: η^2=0.24, p=0.01; NPY: vulnerable vs sham, puncta size, contra: η^2=0.37, p=0.008). Interestingly, there is a moderate or strong effect on vGLUT immunostaining when comparing vulnerable and resilient groups (vGLUT: vulnerable vs resilient, puncta size, ipsi: η^2=0.33, p=0.001, contra: η^2=0.16, p=0.02).

2.4 Differences between vulnerable and resilient groups in molecular markers in the vHPC and BLA

Additionally, in these same animals, we also measured expression of GAD65/67, vGLUT and NPY in two other nuclei important to the control of anxiety, namely the basolateral amygdala (BLA) and ventral hippocampus (vHPC) [218, 231]. Figure 4.5A shows the location of imaging in each of these brain regions.

In the BLA (Sup. Fig. 4.5B), we found that only a few metrics showed an effect of the animal group (vulnerable, resilient or sham). Specifically, we found that vulnerable animals, when compared to resilient (and sham) animals showed a moderate reduction in the total number of GAD 65/67 puncta in the ipsilateral side (Sup. Fig. 4.5B, Fig. 4.5B, GAD column, ANOVA:

F=3.91, p=0.02; post-hoc t-test with FDR correction, p<0.05), and a moderate decrease in the size of GAD puncta in the contralateral side (Sup. Fig. 4.5A; Fig. 4.5B, GAD column; ANOVA: F=4.37, p=0.01, post-hoc t-test with FDR correction, p<0.05). For VGLUT staining, vulnerable animals showed a significant reduction in the total # VGLUT puncta compared to sham controls on the contralateral side (Sup. Fig. 4.5B;Fig. 4.5B, VGLUT column; ANOVA: F=2.24, p=0.02, post-hoc t-test with FDR correction, p<0.05), suggesting a downregulation of VGLUT. However, we found no significant differences specifically between vulnerable and resilient animals on any of the VGLUT metrics (Fig. 4.5A; Fig. 5B, VGLUT column). For NPY staining, vulnerable animals showed a strong increase in the size of NPY puncta compared to resilient (as well as control) animals on the contralateral side (Sup. Fig. 4.5A; Fig. 4.5B, NPY column; ANOVA: F=8.78, p=0.001, post-hoc t-test with FDR correction, p<0.05). Across all markers, there were no differences in the BLA between resilient animals and sham controls on any of the metrics.

In the vHPC (Sup. Fig. 4.5B) as well, we found that there were only a few metrics (across all the markers) that showed an effect of the animal group. Specifically, we found that vulnerable animals, when compared to resilient animals, showed a significant reduction in the intensity of GAD puncta (ipsilateral side; Fig. 4.5C-GAD column; ANOVA: F=3.88 p=0.03, post-hoc t-test with FDR correction, p<0.05) and a marginally significant increase in the intensity of NPY puncta (ipsilateral side; Fig. 4.5C-NPY column; ANOVA: F=2.67, p=0.08, post-hoc t-test with FDR correction, p<0.05). Additionally, vulnerable animals, when compared to sham controls, also showed a reduction in GAD staining (Fig. 4.5C-GAD column; ipsilateral puncta intensity: post-hoc t-test with FDR correction, p<0.05), and a reduction in VGLUT staining e (Fig. 4.5C-VGLUT column; contralateral total intensity: ANOVA: F=3.13, p=0.05, contralateral total #

puncta: ANOVA: $F=3.36$, $p=0.04$, post-hoc t-test with FDR correction, $p<0.05$). Across all markers, there were no differences in the vHPC between resilient animals and sham controls on any of the metrics.

In sum, in the BLA, when comparing the vulnerable versus resilient groups, there was a moderate downregulation of GAD staining and a strong upregulation of NPY staining, but no effects of VGLUT staining. In the vHPC, there was a weak downregulation of GAD, a moderate upregulation of NPY, and no effects of VGLUT.

Together with the results from mPFC, our findings reveal that vulnerability to anxiety outcomes following injury are associated with distinct molecular profiles as compared to resilient injured animals, with stronger overall effects in the mPFC than vHPC or BLA: decreases in metrics of vGLUT and GAD 65/67 staining in mPFC, decreases in metrics of GAD65/67 staining in BLA and vHPC, as well as increases in NPY staining in all three areas. These molecular metrics (total of 17) that specifically exhibit a difference in vulnerable versus resilient and control animals are referred to as molecular indicators of vulnerability.

2.5 Relationship between behavioral and molecular metrics among vulnerable individuals.

The finding of distinct molecular signatures for vulnerability to injury versus resilience motivated us to ask whether, at an individual level, the molecular profiles of the injured animals varied systematically with the extent of vulnerability to anxiety outcomes, across animals. To this end, we identified a key behavioral metric, out of the 11 metrics that constitute an animal's behavioral profile (EZM week 7), and examined its correlation (across animals) with each of the

17 molecular indicators of vulnerability identified in Figures 4.4 and 4.5. Examination of the relationship between this behavioral variable and each of the molecular indicators revealed that most (8/17) showed significant positive correlation across animals (Supplementary Tables 1-3). We then generated a color-coded matrix summarizing the extent of correlation of the various molecular indicators with the behavioral metric (Fig 4.6A). We found that there was a strong correlation between decreases in vGLUT immunostaining in the mPFC (total intensity, puncta number and size), and in the BLA (total intensity), with individual vulnerability. Similarly, there was a strong or moderate correlation between decreases in GAD 65/67 observed in the mPFC (puncta size), in the BLA (puncta size and number), and in the vHPC (puncta intensity), with individual vulnerability. Figure 4.6B illustrates the subsets of metrics and regions where strong correlations between molecular and behavioral measures were observed.

To test if this strong positive correlation was unique to vulnerable animals, of whether the intrinsic variability of metric values within any population of animals would show such a correlation, we repeated this analysis for resilient animals as well as sham controls (Fig. 4.6B, blue and grey dots, respectively). We found that there were no significant correlations between the molecular indicators and behavioral metric for animals in these two groups (Sup. Tables 1-3).

3. Conclusion

In this study, we hypothesized that TBI would lead to a range of anxiety outcomes in an animal model of injury, and that these outcomes would correlate with different molecular signatures. This hypothesis is based on the knowledge that, in clinical TBI, not all patients are

equally affected in terms of neuropsychiatric outcomes, and only about a third will develop anxiety disorders [6].

To test this hypothesis, we combined a dimensionality reduction method, namely, PCA, and a clustering technique, k-means, and we found that TBI animals present two behavioral profiles. Resilient animals did not change their anxiety level in reference to their baseline, and they presented a similar behavior to control animals. Vulnerable animals, on the other hand, significantly increased the exploration of the anxiogenic zone in the behavioral mazes in a consistent manner, indicating a deficit in risk-assessment, or an increase in risk-taking behavior. This change is consistent what has been reported in the literature, that TBI patients, in particular those who suffer moderate to severe brain injury, also present increased impulsivity and compulsive behaviors [241, 242].

We were also interested in exploring molecular markers in anxiety-associated regions of the brain, that may indicate distinctions with resilience and vulnerability. We found neural signatures, in particular in the mPFC of vulnerable animals, indicating that vulnerability could be associated with molecular changes: specifically, downregulation of vGLUT and GAD65/67, and upregulagion of NPY. We found a strong correlation between the extent of behavioral vulnerability, as measured by their performance in the EZM at week 7, and the extent of changes in these molecular metrics for the vulnerable animals. Our results demonstrate that the approach of separating TBI animals based on their behavioral profile is promising and can shed light in anxiety outcomes of TBI in animal models.

Chapter 5: Discussion and future directions

Anxiety outcomes following traumatic brain injury are complex and variable. Whereas suffering from TBI does not always lead to anxiety, the prevalence of this type of disorder is higher among TBI patients than the general population [7]. Thus, animal models of TBI and anxiety offer a valuable opportunity to understand the neural mechanisms of injury that lead to these outcomes and help to develop better clinical treatments. However, the field suffers from a lack of consensus regarding the effects of TBI on anxiety-like behaviors, which complicates the interpretation of the underlying mechanisms of injury that lead to anxiety post-injury. We hypothesized that the inconsistencies are due to factors such as differences (across studies) in injury models and severity, in behavioral assays and metrics, in time-points adopted, and perhaps most importantly, individual differences in brain's response to the injury.

We carried out a series of studies to directly address some of these issues. In chapter three, we adopted a battery of behavioral assays for anxiety and tested animals at several time points over seven weeks. By comparing behavioral patterns across assays and repeatedly testing animals over a long time-course, we were able to identify patterns in how anxiety changed over time and across assays. We also normalized all our behavioral data to each animals' baseline to test the effect of the injury in comparison to individual animals' baseline levels of anxiety. our results pointed to time and task dependence on the effects of TBI in anxiety. We observed an early increase anxiety-like behavior (measured in the EPM and EZM), followed by a late decrease (measured in the EZM and OFT), in mice exposed to CCI. Changes in GABA signaling accompanied these behavioral results, and specifically, TBI animals presented an increased

number and intensity of GAD pixels, as well as an increased number of puncta, suggesting enhanced inhibition in the BLA, associated with the late decrease in anxiety. This study indicated that the effects of TBI, compared to the effects of sham surgery, are not uniform over time nor across multiple behavioral assays. These results suggested that the use of subsets of assays or fixed time points could be a potential explanation for the conflicting results in anxiety outcomes following TBI reported in the literature.

1. Individual vulnerability

We next wondered if there may be a deeper issue at play, especially since TBI is a complex pathology, and even in highly reproducible models, animals can present varying outcomes. Could the variability in each individual's response to injury be an important factor underlying the conflicting and widely variable results in the literature? In chapter four, we directly addressed this issue of variability within TBI animals. Our primary hypothesis was that the injury would affect animals in different ways, with some exhibiting behavioral changes similar to those observed in sham animals, and other showing aberrant behavior. If true, this would imply that comparing a group of TBI animals with highly variable behavioral outcomes, to a group of sham animals with outcomes that overlap substantially may be a flawed approach. An important factor that exacerbates this issue is the standard statistical practice of outlier removal. For a distribution that is highly variable, the removal of 'outliers' can eliminate precisely those individual points that may be the most informative.

To test this hypothesis, we first compared the distributions of anxiety outcomes following TBI versus sham surgery and found substantial overlap between them, as hypothesized. Next, we

developed a quantitative approach that could be applied to our high dimensional behavioral dataset (assays x time-points) to identify distinct behavioral subgroups within the TBI animals. We reduced our high dimensional behavioral data into fewer dimensions that explained most of the variability, and then applied a clustering method to separate TBI animals into two behaviorally distinct groups. We identified that one group of TBI animals was resilient to anxiety changes following injury, since their behavior did not change compared to their pre-injury baseline (nor to sham controls), whereas the other group was vulnerable to anxiety changes following injury: they presented a marked increase in time spent in the dangerous zone of the behavioral tests. Notably, this behavioral vulnerability was associated with molecular signatures in key brain regions; specifically, vulnerable animals presented downregulation of vGLUT and GAD65/67 in the mPFC and BLA, and upregulation of NPY in the mPFC and BLA. These results confirmed our hypotheses that the effects of standardized injury are widely variable across individuals, and that vulnerability to anxiety following TBI has specific neural signatures. Additionally, they shed new light on anxiety outcomes of TBI in animal models: individual variability is a major factor in these outcomes, and accounting for it can yield rich insights into neural mechanisms underlying the outcomes.

We have demonstrated that resilience and vulnerability are fundamental to understanding the anxiety outcomes of TBI. The concept of psychological resilience is not new and has been extensively described in humans, in the context of resilience to psychosocial stress [243-245] and in animal models of stress, in which it has been demonstrated that stress does not affect all animals equally [175, 178, 182]. Resilience, in the context of stress, is defined broadly as the ability of an organism to adapt to adversity, by the engagement of coping strategies [226].

Vulnerability to stress, on the other hand, is the lack of coping strategies and has been implicated in triggering or worsening several psychiatric disorders, such as schizophrenia [246], post-traumatic stress disorder [247], depression [248] and anxiety [249]. Both animal and human literature have started to identify genetic, molecular, and developmental factors that make some organisms more vulnerable to neuropsychiatric disorders than others following exposure to a stressful event. Among the markers for resilience and vulnerability, a subunit of GABAb receptor has been identified as a marker for resilience to stress: knockout animals lacking this isoform were more prone to depression than their counterparts [250]. Resilience also depends on DNA methylation of the neuropeptide corticotrophin-releasing factor (CRF) gene, which is involved in the activation of the neuroendocrine stress response. Chronic stress causes demethylation of this genomic region and leads to a vulnerable phenotype in defeated animals [179]. Early life stress can also induce vulnerability by changing gene expression in the hippocampus and medial prefrontal cortex [251, 252]. Those markers show that resilience is a complex, multidimensional phenomenon, dependent on the interaction of both genetic and environmental factors.

In this context, injury can be considered a ‘stressor’, both physically, due to the damage to the brain, and psychologically, for its emotional effects. It is appropriate, then, to consider how the exposure to the injury will lead to different levels of vulnerability and resilience. Clinical TBI studies do recognize that patients present different levels of vulnerability to developing anxiety as a sequela of injury. However, the role of resilience and vulnerability in the context of TBI has received little explicit attention, with only a few studies have measured patients’ resilience following injury [253-256] [257]. Even fewer studies have (in fact, just one has) applied the concept of resilience and vulnerability to outcomes of TBI in pre-clinical models. In a study of

the long-term cognitive effects of a bilateral frontal CCI, the authors found three distinct groups of animals: some who did not present any level of cognitive deficit (TBI-resilient), a portion who only recovered the full cognitive function after 14 weeks (TBI-vulnerable), and some who never recovered they cognitive function (chronically-impaired) [258]. Although they divided animals in terms of their adaptation to the injury, they did not compare neural markers among these behaviorally distinct groups. This study shows that even for cognitive outcomes, animals will present different degrees of resilience to the harmful effects of the injury. In another study, researchers discussed that the variability in behavioral outcomes following a closed head weight-drop injury in juvenile rats may be due to innate resilience or vulnerability among animals. However, they do not explore this idea in their analysis, i.e., they did not separate the animals into vulnerable and resilient to measure differences between these groups [259]. Additionally, some researchers found that special diets rich in zinc and glucose reduce anxiety- and depressive-like symptoms following TBI [204, 260]. Whereas they refer to the results as resilience, they are better described as improved outcomes due to a specific intervention or therapy, rather than an intrinsic, protective mechanism that affords animals the ability to adapt to the injury and exhibit less severe sequelae. To the best of our knowledge, no previous study has applied the concept of resilience and vulnerability to anxiety outcomes of TBI in animal models.

2. Increased exploration of exposed spaces: adaptive or dysfunctional?

Here, our use of the vulnerability vs. resilience approach revealed that vulnerable animals presented an increased exploration of the exposed (high anxiety) zones within various behavioral arenas following TBI. A challenge in interpreting these behavioral results is that it requires

inferring the animals' emotional state while exploring the mazes. In the specific case of anxiety-like behaviors in rodents, the inference is often ethologically-based: in their natural environment, rodents will engage in two opposing, competing behaviors: exploring a new area and seeking protection [261]. The default interpretation is that when animals spend more time in an exposed zone, they are displaying low anxiety, whereas increased time in the enclosed area indicates high anxiety [262]. This relies on approximating the animal's emotional state in these contexts based on the level of avoidance: high avoidance is taken to signal high anxiety, while low avoidance is taken to signal low anxiety [170]. Consistent with the fact that avoidance of potential threat is a fundamental aspect of human anxiety [263], increased exploration of the exposed zone, i.e., a reduction in anxiety, is often interpreted as an adaptive response, while decreased exploration, i.e., an increase in anxiety, is interpreted as a maladaptive response ('anxiety disorder'). However, engaging in an excessive exploration of a dangerous area can expose animals to unnecessary threats [264], demonstrating lack of behavioral control, and therefore, can itself be maladaptive. In other words, exploration and avoidance are balanced behaviors [265], and a tilting of the balance either towards excessively protective behavior, or towards excessively risky behavior are both plausibly interpreted as dysfunctional behavioral states. Consistent with this view, in clinical studies, reports of patients engaging in risky behaviors following TBI are not uncommon. Addiction, careless and violent behavior, and suicide have been reported following injury [120, 266-268].

Based on these ideas, we concluded that the increased exploration of the exposed zones that we observed in vulnerable animals following injury, is not adaptive, but rather represents a dysfunctional, maladaptive anxiety state. Understanding the mechanisms underlying such

dysfunction after the injury can potentially inform the development of treatments for patients engaging in risk-prone behavior post-injury.

3. Caveats and open issues

In this work, we adopted one focal TBI model, which caused a moderate to severe injury, and in which only about 13% of animals were vulnerable. It would be relevant to test if different injury models and severities alter the level of vulnerability and resilience among animals. Are there specific injury types that increase the chance of animals developing the vulnerable phenotype? Are the neural mechanisms similar or distinct?

Additionally, we only tested male animals. Women often suffer from anxiety-related disorders more often than men [269-271], and although men are twice as likely to suffer a TBI than women [272], women are more likely to suffer from anxiety post-injury [273]. This raises the question of whether women are more vulnerable to suffering from anxiety disorders post-TBI, and whether the mechanism of injury that causes vulnerability is the same in both sexes.

Finally, anxiety dysfunction is only one among the range of affective disorders that commonly affect TBI patients. For example, the prevalence of depression post-TBI is also high [274], and comorbidity between anxiety and depression is not unusual [275]. Our approach of combining different behavioral metrics from TBI animals to identify distinct behavioral groups based on their levels of resilience to the injury, and then investigating associated neural mechanisms, is versatile. The ability of this approach to operate on a multidimensional behavioral dataset, regardless of the number and identity of behavioral assays used or the number of timepoints tested, and still identify relevant behavioral differences among animals, allows for

general applicability. Therefore, this approach can be used readily by researchers to test if other neuropsychiatric disorders that commonly follow TBI have similar or different levels of vulnerability.

Appendix

Supplementary Table 1: Correlation values (rho) and p-values for comparisons between EZM week seven and vGLUT plots that presented a statistically significant difference between vulnerable, resilient and sham animals. Asterisk indicates p<0.05.

vGLUT	mPFC Total px intensity ipsi		mPFC Ave puncta size Ipsi		mPFC Number puncta Ipsi		BLA Total px intensity Ipsi		BLA Total px intensity contra	
	rho	p-value	rho	p-value	rho	p-value	rho	p-value	rho	p-value
control	-0.06	0.8	-0.05	0.84	-0.08	0.76	-0.25	0.34	-0.3	0.27
resilient	0.06	0.72	0.1	0.6	0.05	0.76	0.36	0.057	0.057	0.01*
vulnerable	0.88	0.001*	0.61	0.08	0.8	0.008*	0.89	0.001*	0.80	0.009*

Supplementary Table 2: Correlation values (rho) and p-values for comparisons between EZM week seven and GAD plots that presented a statistically significant difference between vulnerable, resilient and sham animals. Asterisk indicates p<0.05.

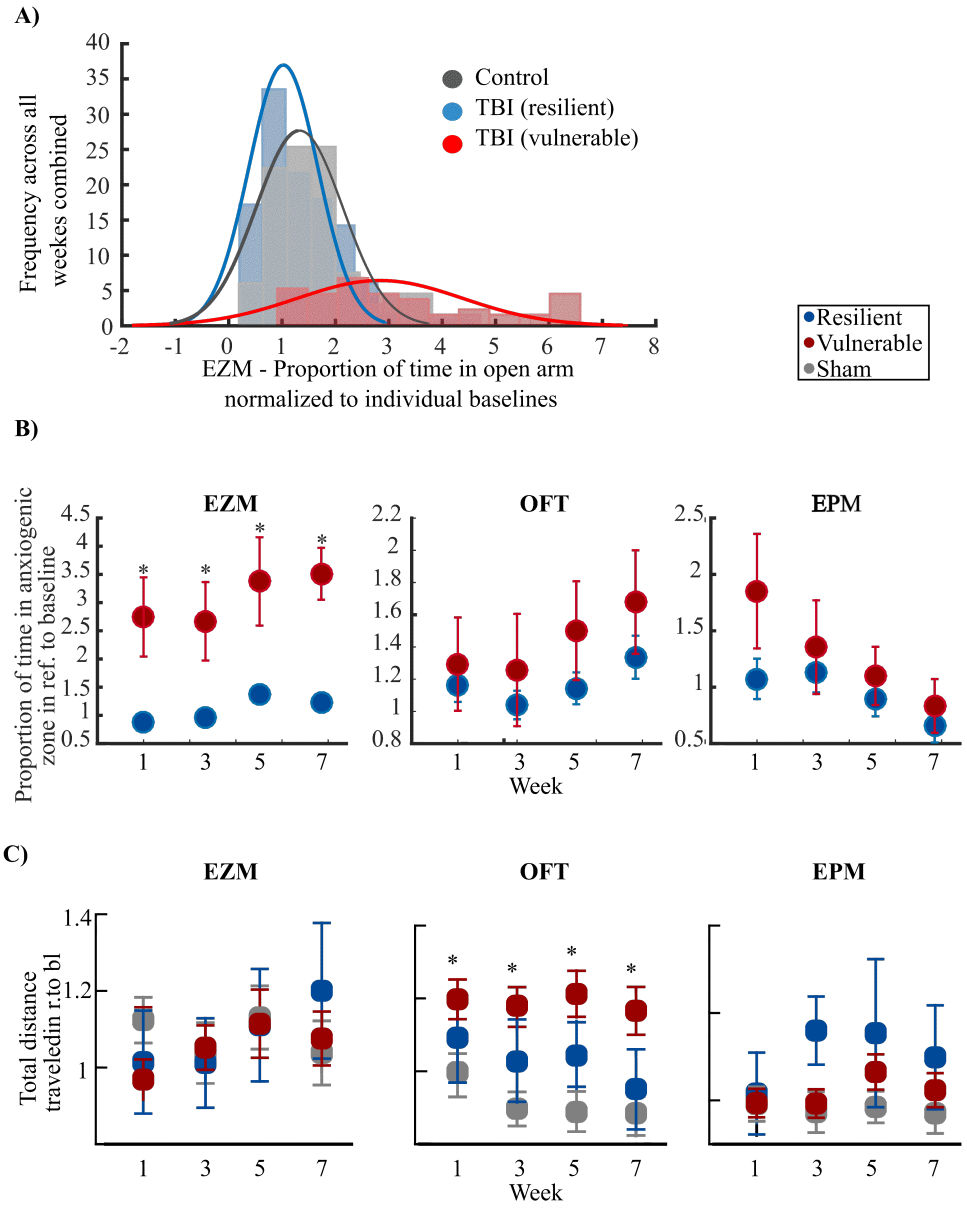
GAD	BLA ave puncta size Ipsi	
	rho	p-value
control	-0.14	0.58
resilient	0.32	0.1
vulnerable	0.70	0.03*

Supplementary Table 3: Correlation values (rho) and p-values for comparisons between EZM week seven and NPY plots that presented a statistically significant difference between vulnerable, resilient and sham animals. Asterisk indicates p<0.05.

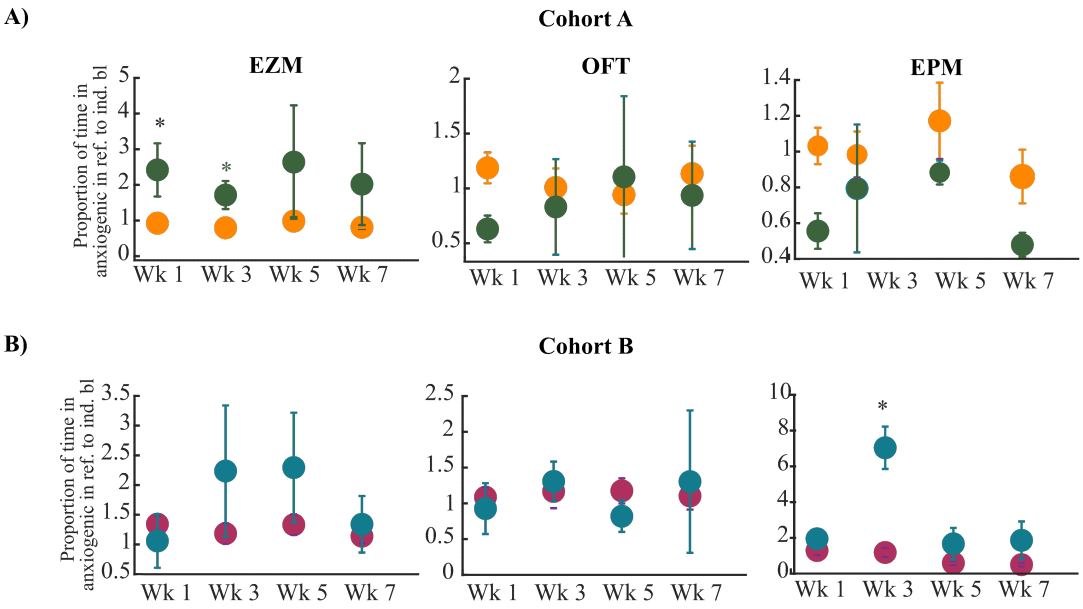
NPY	mPFC Ave puncta size Contra		BLA Ave puncta intensity Ipsi	
	rho	p-value	rho	p-value
control	0.52	0.28	-0.37	0.45
resilient	-0.26	0.28	-0.266	0.26
vulnerable	0.77	0.01*	0.73	0.02*

Supplementary Table 4: contralateral and ipsilateral volume in sham controls, resilient and vulnerable animals. A three-way ANOVA shows no main effect of treatment in the contralateral side ($F=1.52$, $p=0.23$), and a main effect of treatment in the contralateral side ($F=4.42$, $p=0.019$), and TBI animals (resilient and vulnerable) presented a significant decrease in ipsilateral hemispheric volume compared to sham controls ($p<0.05$). Resilient and vulnerable animals did not differ from each other.

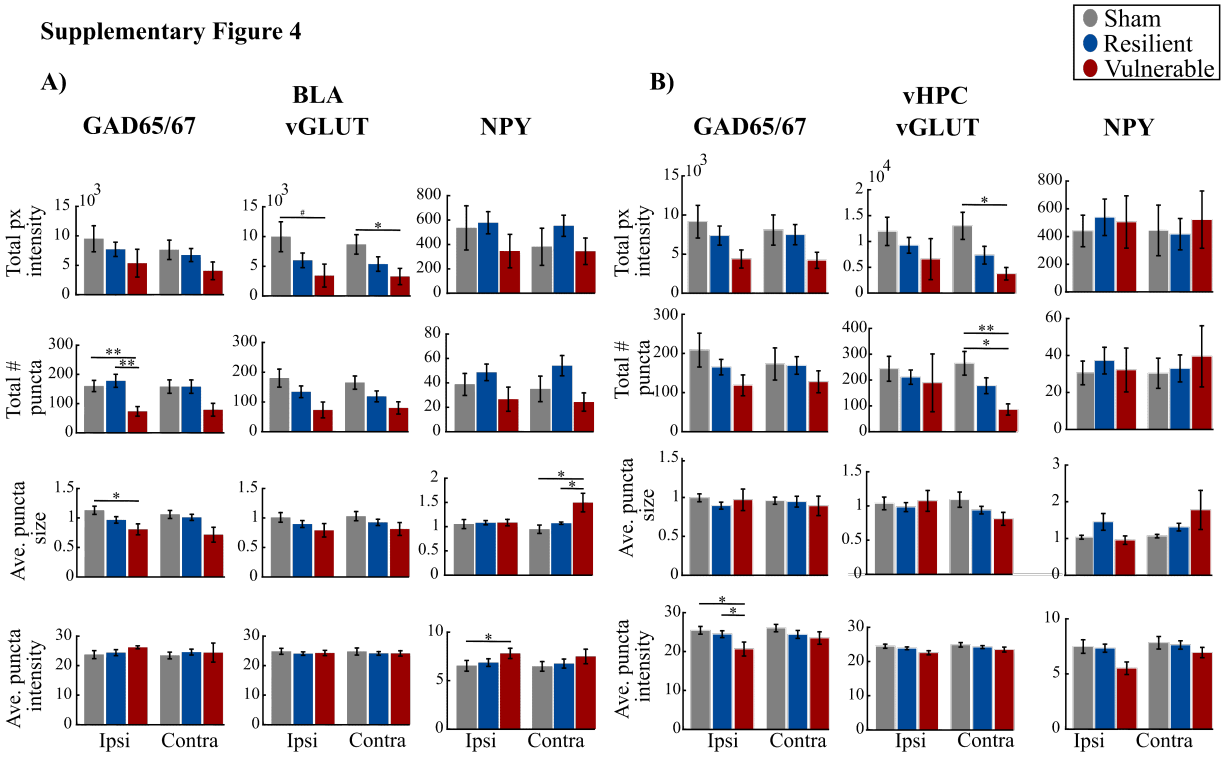
	Mean contralateral volume (e+07)	SEM contralateral volume (e+06)	Mean ipsilateral volume (e+07)	SEM ipsilateral volume (e+06)
Sham controls	2.3	2.8	2.2995	2.9436
Resilient	1.65	2.44	1.2873	2.1918
Vulnerable	1.64	5.14	1.2708	4.0155



Supplementary Figure 4.2: A) Histogram showing the proportion of time in the EZM across all time-points (x-axis) and the frequency of occurrences (y-axis), separating animals into sham, resilient and vulnerable. B) Proportion of time resilient and vulnerable animals from cohort B spent in the anxiogenic zones in the EZM, OFT and EPM. There was a main effect of treatment in the EZM ($F=56.34$, $p>0.001$), and a post-hoc effect on weeks one, three, five and seven. There was a marginal main effect of treamtne in the OFT ($F=2.08$, $p=0.1$), with no post-hoc effect. There was no main effect of treatment in the EPM ($F=1.43$, $p=0.24$). C) Total distance travelled in the mazes, combining cohort A and B. There was a main effect of treatment in the OFT (center, $F=29.74$, $p=0$,) with vulnerable animals traveling more than sham animals on weeks one, three and five ($p<0.05$), and in the EPM ($F=3.84$, $p=0.02$), with no post-hoc effect.



Supplementary Figure 4.3: proportion of time in anxiogenic zone for sham controls, separated by cohort A and cohort B. In the EZM, there was a main effect of treatment for Cohort A (A, $F=40.48$, $p<0.0001$) and cohort B (D, $F=4.36$, $p=0.004$), with a post-hoc effect on weeks one and three for cohort A. In the OFT, there was no main effect of treatment for neither cohort A (B, $F=0.58$, $p=0.44$) or cohort B (E, $F=0.003$, $p=0.85$). In the EPM, there was no main effect of treatment for cohort A (C, $F=2.87$, $p=0.09$), and there was a main effect of treatment for cohort B (F, $F=60.63$ $p<0.001$), with a post-hoc effect on week three.



Supplementary Figure 4.4: Pixel and puncta analysis in the BLA (A) and vHPC (B), for GAD65/67, vGLUT and NPY. Bars represent SEM. Data from 53 animals (vulnerable=9, resilient=28, sham=16), including outliers. *: $p<0.05$ and **: $p<0.01$, for difference between groups by post-hoc t-test with false discovery rate (FDR) correction.

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Biographical Statement

Juliana Popovitz was born in the city of Curitiba, in the southern part of Brazil. She attended Federal University of Parana, where she graduated in Psychology. During her undergraduate studies, she started becoming interested in academic work, and join the Behavior Analysis Lab. There, she researched parental styles and how they affect children's academic performance. Her deep interest in mental health led her to later pursue a Master's degree in Clinical Psychology, developing psychotherapy protocols to treat depression and anxiety. During this time, she also worked as a clinical psychologist in her hometown. By the time she finished her Master's degree, she decided to undertake a new challenge: pursuing a PhD in Psychological and Brain Sciences, at Johns Hopkins University. During her PhD, she studied the effects of traumatic brain injury in anxiety-like behaviors in an animal model, and developed a novel approach to understanding how injury affects individuals in different ways.